ENDOCRINOLOGY

VOLUME 66 JUNE, 1960 NUMBER 6

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RADIOIODINE UTILIZATION IN THE CHICK[1]

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ABSTRACT

The effects of thyrotrophin, thiouracil and diets containing various amounts of iodine upon thyroidal metabolism of radioiodine were studied in chicks. Great variability of quantity of radioiodine accumulated by the chick thyroid at different seasons was redemonstrated. The level of additional administered iodine did not appreciably affect this variability. TSH increased the absolute thyroid uptake of I^{131}; additionally administered iodine and thiouracil depressed it.

Labeled monoiodotyrosine, diiodotyrosine, triiodothyronine, and thyroxine were found in normal chicks. Their proportions were altered by the various experimental conditions and by season alone. Thyrotrophin favored thyroxine production. Thiouracil appeared to block hormone synthesis at the monoiodotyrosine stage. With time, MIT/DIT ratios of more than seven were progressively developed, indicating accumulation of MIT in thiouracilized thyroids. Some of the factors in the well known seasonal variability of thyroid function in chicks are discussed.

STUDIES of the utilization of radioiodine by thyroids of cyclostomes, fishes, amphibians, reptiles, and mammals have generally revealed a surprisingly uniform pattern of biochemical events (1). However, because information was lacking until recently, it has been impossible to make generalizations which would include the avian thyroid. This is surprising in view of the popularity of use of the newly hatched chick in bioassay of thyrotrophic hormone. In two recent reports of research utilizing radioiodine, I^{131}, Shellabarger and Pitt-Rivers (2) have noted the production of triiodothyronine by chick thyroids, and Vlijm (3) has described the partition of I^{131} among the known iodothyronine and iodotyrosine fractions in normal and goitrogen-treated chicks. Among the many questions left unanswered by these two studies is the relation of iodine metabolism in the chick thyroid to total iodine intake, and the effect of thyrotrophic stimula-

Received July 1, 1959.

[1] This study was aided by a grant from the National Science Foundation.

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tion on I^{131} metabolism. The work reported here deals with these two questions. In addition, we have addressed ourselves to the problem of extreme variability in response of the thyroid of the day-old chick. This type of variability is often so great that large groups of chicks must be used to obtain statistically valid results.

MATERIALS AND METHODS

The 3 sets of experiments reported here were done with 3 different groups of White Leghorn cockerels[3] and at different times. Since considerable variation was found from one experiment to the next, and it was possible that the variation was due to dietary and temperature differences experienced by chicks before they came to our laboratory

TABLE 1. PLAN OF EXPERIMENTS

Experiment	Month used	Groups of animals	Temperature	Basal diet	Age at the time of I^{131} injection	Dose of I^{131}
1	September	Normal chicks	35° C (in chick brooder)	Purina Super Chick Startena	2 days	5 μc.
2	November	1. Control 2. Birds fed thiouracil (0.05%) from 9 days old until sacrifice	Room temperature (25–30° C)	Purina Super Chick Startena from 2 days old to 8 days old; after this, low iodine diet	17 days	10 μc.
3	January	1. Control 2. Birds injected daily with KI-solution from 9 days old until the day before sacrifice 3. Birds injected daily with TSH from 13 days old until the day before sacrifice	Room temperature (25–30°C)	Purina Super Chick Startena from 2 days old to 8 days old; after this, low iodine diet	17 days	10 μc.

at 2 days of age, in 2 experiments they were kept in the laboratory for periods of "adaptation" before beginning experiments. Details of planning of each of the 3 experiments are summarized in Table 1.

Two basal diets were used, a commercial chick starter mash "Purina Super Chick Startena,"[4] and the low iodine test diet.[5] Both diets and water were available *ad libitum* and were freely consumed. The basal diets were altered in the instances specified in Table 1 by addition of thiouracil powder to a concentration of 0.05%. Iodide was given by daily subcutaneous injection of 30 μg as the sodium salt. Purified beef thyrotrophic hormone (TSH)[6] was injected subcutaneously, 0.5 mg. per day, from 4 days preceding I^{131}-injection until the day before sacrifice. The radioiodine was given tracer-free as iodide by intraperitoneal injection of 5 or 10 μc. Chicks were killed in groups at intervals up to 120 or 168 hours after radioiodine administration with chloroform and their thyroids were dissected out immediately. The thyroids were individually homogenized in 0.9% saline solution. An aliquot of the solution was taken for measurement of total thyroidal radioactivity in a well-crystal scintillation detector. An aliquot of the original radioiodine solution was counted at the same time and the degree of thyroidal iodine

[3] All the chicks of the three experiments were purchased from Hall Brothers Hatchery, Wallingford, Connecticut.

[4] Purina Super Chick Startena contains 0.5% iodized salt.

[5] Low-iodine test diet was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

[6] Thyrotrophic hormone is a gift of Parke, Davis Company. Its assayed strength was 10 J.-S. units per mg.

accumulation could then be expressed in terms of per cent of the original injected dose. After radioactive iodine measurement the homogenates of each group of chicks were extracted together with saline solution and prepared for paper chromatographic analysis by the method described by Lissitzky (4). This procedure, briefly, involves enzymatic hydrolysis of the aqueous saline extract of thyroid. An acid butanol extract of this hydrolysate is reduced to dryness below 45° C and a butanol solution of the residue is applied to paper for chromatography with butanol-ammonia, or butanol-acetic acid solvents. Known compounds of iodine which were used to identify the unknown radioactive iodine chromatographic spots included potassium iodide (KI), monoiodotyrosine (MIT), diiodotyrosine (DIT), 3,5,3′-triiodothyronine (TITN), and thyroxine (Tx). The distribution of radioactivity along the length of the filter paper chromatograms was determined with an end-window Geiger tube and recorded on graphs. The total radioactivity of chromatograms was expressed in terms of the numbers of ruled mm. squares on the graph under the activity curve. The numbers of squares under the activity curves of iodide or the several radioiodinated amino acids were counted separately, and the percentage of radioactivity of each of these compounds to total radioactivity was calculated.

RESULTS

Experiment 1. Normal chicks. There was much individual variation in the uptake of I^{131} by the thyroid gland (Table 2). At maximum only 1.10% of the injected dose was accumulated by the thyroid; this was observed in a bird killed at 48 hours after the isotope injection.

For chromatography, because of the low uptake, the thyroid glands of the birds of two groups, killed at two successive time intervals, were homogenized together. The birds sacrificed at 168 hours after I^{131}-injection were not used for this analysis. In the thyroid glands of the birds killed at 6 and 12 hours after I^{131}-injection, most (79%) of the radioiodine was found as MIT and DIT (Table 3), in approximately equal proportions. A small amount of radiothyroxine was detected, but no TITN. Almost the same results were obtained in the birds of other groups killed later, but TITN and one unknown substance were detected in trace quantities in these birds. The Rf of the unknown substance was about 0.44 in butanol-ammonia solvent.

Experiment 2. Effect of a low-iodine diet and thiouracil on radio-iodine metabolism. Although individual variation was large, it was clear that the maximum thyroidal uptake of radioiodine took place by 6 hours after administration of the isotope both in thiouracil-treated and control birds (Fig. 1). Thiouracil inhibited the uptake of radioiodine by the thyroid gland. In the

TABLE 2. UPTAKE OF RADIOIODINE BY THE THYROID GLANDS OF NORMAL CHICKS FED PURINA SUPER CHICK STARTENA, EXPRESSED AS PER CENT OF TOTAL DOSE; STANDARD ERROR INDICATED, EXPERIMENT 1

Hours after I^{131} injection	6	12	24	48	72	96	168
Number of birds killed	5	5	5	5	5	5	5
Uptake	0.25 ± 0.071	0.35 ± 0.178	0.24 ± 0.047	0.44 ± 0.182	0.32 ± 0.113	0.26 ± 0.087	0.13 ± 0.056

TABLE 3. RELATIVE PROPORTIONS OF RADIOIODINE COMPOUNDS IN THYROID GLANDS, EXPRESSED AS PER CENT OF TOTAL EXTRACTABLE RADIOACTIVITY FROM THE GLANDS. EXPERIMENT 1

Birds used	Iodide	MIT plus DIT	I^{131} in MIT / I^{131} in DIT	Tx	TITN	Uknown substance
Birds killed at 6 and 12 hours after I^{131}-injection	1.9	78.6	1.1	1.9	—	—
Birds killed at 24 and 48 hours after I^{131}-injection	1.8	83.4	1.0	4.4	0.9	2.0
Birds killed at 72 and 96 hours after I^{131}-injection	2.2	67.6	1.0	5.6 (Tx+TITN)		1.8

birds fed the low iodine diet thyroidal radioiodine fell rapidly from the 6-hour peak (30%) so that only about half of the peak value remained at 24 hours after I^{131}-injection. The rate of decrease of thyroidal radioiodine was similar in both groups (Fig. 1).

Radiochromatographic analysis revealed that in the thyroid glands of the thiouracil-treated birds, most of the radioiodine (74–83%) was present as MIT and DIT. Radioactive MIT greatly exceeded DIT (Table 4). The ratio MIT/DIT which was 2.4 at 6 hours after I^{131}-injection, increased to

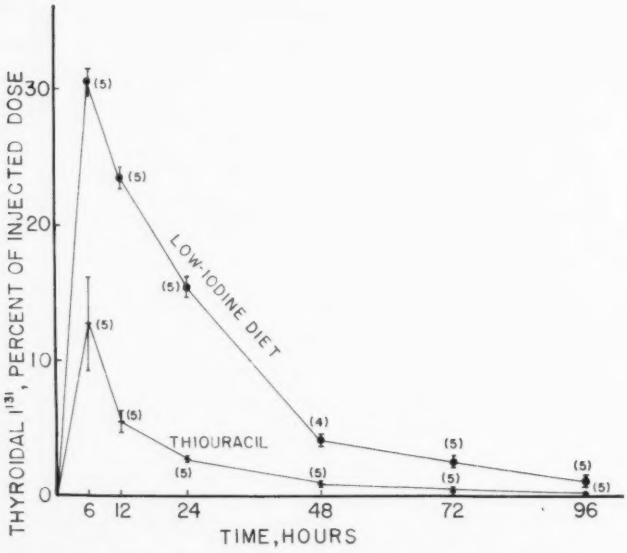

FIG. 1. Thyroidal radioiodine accumulation and loss in thiouracil-treated chicks and controls fed a low iodine diet. Experiment 2. Numbers in parentheses indicate numbers of animals represented by each point. Vertical lines show standard error.

TABLE 4. RELATIVE PROPORTION OF RADIOIODINE COMPOUNDS IN THYROID GLANDS, EXPRESSED AS PER CENT OF TOTAL EXTRACTABLE RADIOACTIVITY FROM THE GLANDS. EXPERIMENT 2

Group	Time intervals killed after I^{131}-injection	Iodide	MIT plus DIT	I^{131} in MIT / I^{131} in DIT	Tx	TITN	Unknown substance
Thiouracil-treated birds	6 (5)*	0.7	83.2	2.4	1.7	—	—
	12 (5)	—	80.4	4.1	—	—	—
	24 (5)	—	83.2	3.3	—	—	—
	48 (5)	0.4	73.5	7.3	—	—	—
Control birds fed low iodine diet	6 (5)	1.4	62.8	1.7	14.1	0.6	0.5
	12 (5)	2.4	64.3	1.2	13.3	2.4	1.4
	24 (5)	4.6	55.5	1.5	18.5	4.6	4.6
	48 (4)	4.2	56.9	1.7	21.0	2.6	1.1
	72 (5)	5.6	61.7	1.6	10.3	4.6	5.0

* Numbers in parentheses indicate number of chicks in each test.

7.3 at 48 hours. Radioiodide and radiothyroxine were present in trace quantities. The unknown substance was not detected. In the birds fed the low-iodine diet most radioiodine (57–64%) was found also in the MIT and DIT fractions. The total of radioactive MIT and DIT was greater in percentage in the thiouracil-treated birds than in controls. Radio-MIT exceeded radio-DIT, but to a much smaller extent than in thiouracil-treated birds. The ratio of MIT/DIT varied from 1.5 to 1.7. Tx (10–21%) and TITN (1–5%) were found in the birds killed at all intervals. An unknown substance was detected in small proportions (0.5 to 5.0%). Its Rf in butanol-ammonia solvent was 0.48.

Experiment 3. Effect of TSH and KI on radioiodine metabolism. The uptake by thyroid glands was greatly increased by pretreatment with TSH (Fig. 2). The maximum uptake in TSH-treated birds was about 15% of the injected dose, and it took place by 6 hours after I^{131} injection. It was not as rapidly lost from the thyroid as in Experiment 2. At 24 hours after injection about 13% of the injected I^{131} was still held by the gland. After that the decrease of I^{131} in the thyroid glands was more rapid. At 48 hours after the injection only 4.7% of the injected dose (31% of the peak value) remained. The maximum uptake by the glands of control birds fed the basal low-iodine diet occurred by 6 hours after I^{131}-injection (Fig. 2) and it was less than one-seventh (about 2% of the administered radioiodine) of the value for TSH-treated birds. Thyroidal radioiodine of low-iodine diet chicks decreased to about half of the peak quantities at 24 hours after I^{131}-injection.

In the birds injected with KI, the accumulation of I^{131} by thyroid glands was inhibited and there was much individual variation (Table 5). The maximum thyroidal uptake was 0.36% in one bird killed at 12 hours after I^{131}-injection.

TABLE 5. UPTAKE OF RADIOIODINE BY THE THYROID GLANDS OF KI-INJECTED CHICKS, EXPRESSED AS PERCENT OF TOTAL DOSE. EXPERIMENT 3

Hours after I^{131} injection	6	12	24	48	120
Number of birds killed	4	4	4	4	4
Uptake	0.15 ± 0.068	0.16 ± 0.074	0.21 ± 0.033	0.14 ± 0.042	0.07 ± 0.022

In all three groups of birds in this experiment, most of the radioiodine was present in MIT and DIT fractions (Table 6). The ratio of radioactive MIT to DIT in contrast to the earlier experiments, was always less than 1.0. In addition to stimulating a greater total thyroidal I^{131}-uptake (Fig. 2), TSH appeared to stimulate formation of slightly greater proportions of radio-Tx (Table 6). Furthermore, the peak in radiothyroxine formation occurred much earlier in TSH-treated chicks than in controls or KI-injected animals. Labelled TITN was found in all groups, in almost equivalent proportions (0.8 to 3.7%). Iodide and one unknown substance (Rf in butanol-ammonia: 0.40) were detected in small quantities in the three groups.

DISCUSSION

One of the most serious drawbacks in the use of the thyroid response of newly-hatched chicks for bioassay of thyrotrophic hormone is its variability. To overcome this, the use of large groups of untreated control birds has been recommended (5), as well as more involved techniques such as pretreatment with propylthiouracil and thyroxine (6). Another type of vari-

TABLE 6. RELATIVE PROPORTIONS OF RADIOIODINE COMPOUNDS IN THYROID GLANDS, EXPRESSED AS PER CENT OF TOTAL EXTRACTABLE RADIOACTIVITY FROM THE GLANDS. EXPERIMENT 3

Group	Time intervals killed after I^{131}-injection	Iodide	MIT plus DIT	$\frac{I^{131} \text{ in MIT}}{I^{131} \text{ in DIT}}$	Tx	TITN	Unknown substance
Birds injected with KI	6 (4)*	3.9	68.3	0.6	9.7	2.6	3.9
	12 (4)	3.4	71.3	0.7	6.8	3.4	2.6
	24 (4)	2.7	70.6	0.7	8.0	1.2	6.2
	48 (4)	—	75.1	0.4	13.7	—	1.6
	120 (4)	1.9	61.2	—	16.7	1.6	2.5
Birds injected with TSH	6 (5)	1.4	64.5	0.6	18.1	0.8	—
	12 (4)	1.4	59.8	0.5	15.1	1.7	0.9
	24 (4)	1.0	59.7	0.7	8.8	0.9	—
	48 (4)	1.8	64.8	0.4	8.1	1.8	—
	72 (4)	3.6	60.0	0.4	12.7	—	—
	120 (4)	—	66.0	0.7	9.3	—	—
Control birds fed low iodine diet	6 (4)	1.2	66.7	0.9	4.0	2.0	5.6
	12 (5)	1.7	66.5	0.9	8.0	—	4.7
	24 (4)	1.4	59.7	0.7	9.0	2.4	4.9
	48 (5)	2.2	65.1	0.4	8.9	3.7	2.9
	120 (5)	—	61.8	—	14.7	—	—

* Numbers in parentheses indicate numbers of chicks in each test.

ability appears to be seasonal (7), but no particular predictable pattern seems to be characteristic of seasonal variability in the sensitivity of the chick's thyroid. Many possible explanations for the apparently capricious reactivity of the chick's thyroid present themselves, but none has been thoroughly investigated. Among these possibilities are differences a) in the hen's or chick's diet, b) in degree of yolk resorption, c) in temperature experienced between hatchery and laboratory, d) genetic stock. It is worthy of note that Bates and Cornfield (6) found in New Hampshire Red chicks fed a completely synthetic diet that the maximum thyroidal uptake of I^{131} from week to week varied between 20% and 60%. Frey and Albert (8) found no difference in thyroidal uptake of I^{131} between chicks fed a commercial diet and others that were fasted.

Our own observations, summarized in this paper, confirm the reports of various authors concerning the individual and seasonal variations in quantitative aspects of thyroidal function. Beyond this, our data show that this variability extends into the qualitative aspects of thyroidal biosynthesis of hormone.

It is clear that some of the variability in radioiodine accumulation by newly hatched chicks can be explained on the basis of diet. In Experiment

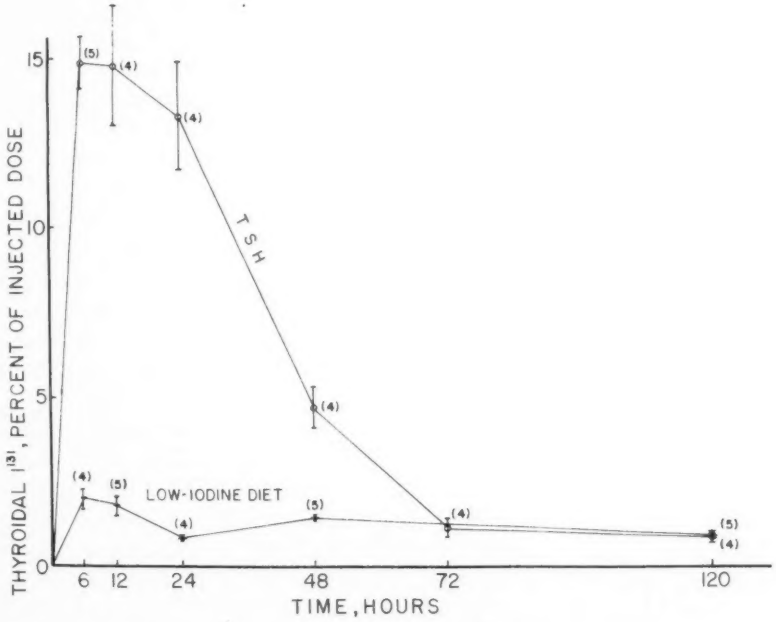

FIG. 2. Thyroidal radioiodine accumulation and loss in TSH-treated chicks and controls fed a low iodine diet. Experiment 3. Numbers in parentheses indicate numbers of animals represented by each point. Vertical lines show standard error.

3 the daily injection of 30 μg. of iodide depressed the maximum thyroidal radioiodine uptake of 17-day old chicks by a factor of about 8 (Fig. 2, Table 5) below the maximum uptake by chicks fed a basal low-iodine diet. Comparably low thyroidal uptakes (less than 1%) were seen in only one other experiment in 2-day old chicks fed a commercial diet which contained iodized salt. It would be tempting to conclude that low thyroidal uptakes of radioiodine in chicks are due to dietary dilution of the isotope by large amounts of stable iodine, or to deposition of large amounts of iodine in the yolk by hens on a high iodine diet (9). The statement by Frey and Albert (8) that starved chicks and chicks fed a commercial mash have similar thyroidal I^{131} uptakes contradicts this conclusion. However, since our data agree with the well established reduction of thyroidal I^{131}-uptake by augmented dietary or environmental stable iodine in other vertebrate forms (11, 12), it seems reasonable to conclude that variations in dietary iodine of the chick, or its mother, may be the basis of at least some of the observed variability in thyroid function.

Thyrotrophic hormone produced a seven-fold increase in the peak value of radioiodine accumulation by the thyroid (2% increased to 15%) (Fig. 2) which agrees with the well known stimulation of thyroid weight (5) and histological structure (13) used as the basis of several bioassay procedures. TSH in Experiment 3 (January) increased the rate of loss of I^{131} from the thyroid beginning 24 hours after I^{131} injection (Fig. 2) compared with controls fed a low-iodine diet. This would support the recommendation of Bates and Cornfield (6) and Piotrowski *et al.* (7) that radioiodine depletion can be used as the basis for a TSH-bioassay procedure. However, in Figure 1, which summarizes an experiment completed in November, it is clear that untreated chicks in another season may have an even higher thyroidal I^{131} uptake (30%), while fed the basal low-iodine diet than the TSH treated birds. Furthermore, the rate of loss of I^{131} in November is even more rapid in untreated chicks than it is in TSH-treated chicks in January. Since both were fed the basal low iodine diet, an attractive explanation of the seasonal difference in this case is a difference in endogenous TSH secretion, whether basically related to environmental temperature, light, or other experiences of the experimental birds. Other factors are not excluded, of course, including the possible seasonal variation in the maternal contribution to the composition of the egg (10). This type of discussion opens many more questions than it settles, and all of these questions are susceptible to experimental analysis. It is hoped that some of the suggested research will be undertaken. One of the most striking facts which this study has yielded is that the "natural" seasonal variation in quantitative radioiodine uptake in thyroids of chicks may be greater (15-fold) than any induced by the various experimental procedures (7- or 8-fold).

Seasonal variability in thyroid function extends also to the pattern of biochemical utilization of the iodine (Tables 3, 4, 6). In the first (Septem-

ber) experiment in which the I^{131}-uptake was the lowest, radio-thyroxine formation was much slower and reduced in proportion (fraction of total thyroidal radioactivity) when compared to the second and third experiments. In the latter two experiments the maximal proportions of radiothyroxine were 21.0% and 14.7%. It is to be noted that the highest proportion of radiothyroxine formed in untreated chicks from the radioiodide was in those chicks with the highest thyroidal accumulation of I^{131}. Also the largest TITN fraction (4.6%) was found in this group of birds. Thiouracil, as was to be expected (3) blocked the formation of thyroid hormone (Table 4), TSH accelerated it in time and slightly stimulated it in extent (Table 6). Injection of iodide, although it depressed uptake, did not appear to affect appreciably the proportion of I^{131} found in hormonal form. With the exception of the thiouracil block, it is again impressive to note that seasonal variations in thyroxinogenesis may be much greater than variations induced experimentally by TSH or iodide treatments.

One respect in which the normal chick thyroid is unusual is in the relatively high proportion of MIT. This was noted by Taurog, Tong, and Chaikoff (14) in an adult hen, and by Vlijm (3) in young cockerels about 10 weeks old. In our three experiments the ratio MIT/DIT was high (ca. 1.0) in the September experiment, higher in November (1.2 to 1.7), and reduced in January (0.4 to.0.9). There was no clear tendency, as reported by Vlijm (3), for higher ratios earlier, and lower ratios later, in a given experiment as one might expect if MIT were a precursor for DIT. TSH and iodide treatment had either no effect, or slightly favored lower ratios, as might possibly be expected from the work of Leloup and Lachiver (15). These authors claimed that in the rat a deficiency in dietary iodine favored the production of those iodotyrosines (MIT) and iodothyronines (TITN) with the smaller number of iodine atoms.

It is of particular interest that after thiouracil feeding ratios of radioactive MIT to DIT up to 7.3 were found. The value of this ratio increased from 2.4 at 6 hours after I^{131}-injection to 7.3 at 48 hours. Thus, it would appear that in the chick thiouracil produces a block in thyroxine synthesis between MIT and DIT. The progressive accumulation of labeled MIT due to this block leads to the growth in the MIT/DIT ratio (Table 4). In the rat, where this question has been studied intensively, it has been believed that the metabolic block provided by thiouracil is at the step of oxidation of iodide (16). However, recently, Slingerland *et al.* (17) have studied this same question in the rat and found that increasing concentrations of dietary propylthiouracil, up to 0.1%, produced radioactive MIT/DIT ratios which increased with the dose up to a value of 15.0. Measurements of the MIT/DIT ratios made by Slingerland all were at 24 hours after radioiodine administration. It is quite possible that if these workers had studied the phenomenon as a function of time after I^{131} injection the data for the rat would show the gradual accumulation of radio MIT as we observed it

in the chick. Thus, our information derived from the study of the birds' thyroid confirms the even more striking results of Slingerland *et al.* suggesting that thiouracil and propylthiouracil can block the iodination of MIT to form DIT.

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EFFECTS OF CORTISONE UPON BLOOD, ADRENAL CORTEX, GONADS, AND THE DEVELOPMENT OF OSTEOPOROSIS IN BIRDS[1]

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ABSTRACT

Domestic birds exhibited a high tolerance for massive doses of cortisone and developed osteoporosis that was very slight in the rooster, moderately severe in the capon, and severely destructive in the hen. After four weeks of treatment, the adrenals and gonads were atrophied, and there was hypercalcemia, associated with an elevation in the ultrafilterable fraction of the serum calcium. Egg production generally ceased within one week, but a few birds continued to lay irregularly, or briefly, and produced shell-less dwarf eggs with a membranous stalk. Bone was resorbed from the endosteal surface of the cortex of the skeleton by means of a local mechanism that did not require formation of osteoclasts. Estrogen injected into cortisone-treated birds produced the phosphoproteinemia, phospholipidemia, and lipema (associated with twenty-fold rise in the non-ultrafilterable fraction of the serum calcium), the same as in normal birds. Estrogen, without androgen, did not give the hen protection against osteoporosis. The rooster was relatively resistant to osteoporosis.

OSTEOPOROSIS is a physiopathological disorder of the skeleton found in White Leghorn hens bred for heavy egg production. It appears to be a disorder of bone influenced by endocrine factors, but the pathologic mechanisms, metabolites, and metabolic pathways leading to its development are obscure. How cortisone accentuates osteoporosis in a susceptible animal is of special interest.

The literature on the action of cortisone on bone metabolism emphasizes the relationship between the ability of this steroid to inhibit protein synthesis and retardation of growth of connective tissue. Karnofsky, Ridgeway, and Patterson (1) found that cortisone acetate inhibited growth of the developing chick embryo. Ruskin, Pomerat, and Ruskin (2) performed similar experiments on explants of avian tissues and concluded that the inhibitory action was due to a direct effect of the cortisone on the cells. Kudzia and Champion (2) found that cortisone in doses as low as 2 mg. per kilo. reduced body weight and egg production in domestic hens. Pick and Stammler (4) measured body weight and length of the birds and con-

Received July 7, 1959.

[1] This work was aided by grants from the Easter Seal Foundation and the Society for Crippled Children and Adults, the Josiah Macy, Jr. Foundation, Squibb Institute for Medical Research, and Ayerst Laboratories, Inc.

cluded that hydrocortisone produced significant retardation of growth despite normal or even increased food intake. Buno and Goyena (5) observed suppression of growth of explanted limb buds *in vitro*. Dulin (6) found that the cockerel was more resistant than the capon to the growth-inhibiting effects of cortisone, and attributed this to the protective effect of endogenous androgen. He also found that cortisone inhibited development of the testes without any appreciable reduction in the weight of the pituitary, and, therefore, possibly without diminishing the secretion of gonadotropin. Kowalewski measured the uptake of radiosulphur in the bones of cortisone-treated cockerels and found markedly lowered synthesis of sulfated components of connective tissue (7–8); this was counteracted by simultaneous administration of 17-ethyl-19-nor-testosterone but not by methyltestosterone or testosterone propionate.

Montgomery (9) injected large doses of cortisone, 5 mg. per day, in chicks for 18 days and produced extraordinary stunting of growth. Hublé (10) found that this was due to inhibition of endochondral ossification and, consequently, failure of bones to grow in length. Brown, Meyer, and Brown (11) adrenalectomized roosters and found that cortisone acetate maintained the body weight and blood electrolytes, total nitrogen, and sugar at normal levels.

Follis (12) and Sissons and Hadfield (13) described nonspecific inhibition of bone growth by cortisone in mice, rats, guinea pigs, and rabbits. In mice, Gluckman and Shklar (14) observed that osteoporosis was produced by cortisone and prevented with estrogen. Sobel and his associates (15) concluded that cortisone in rats caused preferential resorption of the labile fraction of connective tissue protein (hexosamine-rich fraction) without disturbing the metabolically inert mature collagen of connective tissue. Clark *et al.* (16) injected Ca^{45} in cortisol-treated rats and observed low absorption but no catabolic effects on bone calcium. The effects of cortisone on bone appear to vary with dose, age, sex, and species. The rat is relatively resistant to osteoporosis. Birds are apparently susceptible. The object of this investigation is to determine the nature of the factors responsible for susceptibility and resistance to osteoporosis, and the response of the avian skeleton to cortisone treatment.

MATERIALS AND METHODS

Capons, roosters, and hens were observed before and after treatment with massive doses of cortisone, and for various periods, as will be described in a running account of the experiments. The bones were cut in cross sections grossly on a band saw and examined by macro and microradiographic, routine histological and histochemical, methods. The left ovary and both adrenals were weighed wet. The composition of the blood was examined by methods described in previous publications (17–19). Hexosamine was measured by the method of Boas (20) and collagen was measured by the method of Neuman and Logan (21).

RESULTS

Control roosters. The range of the levels of eleven constituents of the blood of 10 control roosters is shown in Table 1, Column II. The range of weight of the adrenals and gonads is shown in Table 2. Microradiograms were more informative than histological sections. A cross section of the cortex of the tibia, 2.0 cm. distal to the knee joint, showed a homogeneous structure with primary Haversian, or solid, compact lamellar bone and a few secondary Haversian systems. The Haversian canals were small and uniform in size, 20 to 40 μ in diameter. The periosteal surface was relatively avascular (Figs. 1A and 3A).

Control hens. The composition of the blood of 10 laying hens is shown in Table 1, Column III. The range of the weight of the adrenals and ovaries is shown in Table 2. Egg production varied from 14 to 21 during a four-week test period. The tibia of the laying hen, compared with that of the rooster, showed a thinner cortex, many resorption cavities, Haversian canals enlarged to 200 to 500 microns in diameter, and definite osteoporosis. The hexosamine content of the compact bone, as shown in Table 3, was also somewhat lower than that of the rooster. These observations, similar to those reported previously (19), indicate that modern laying hens bred

TABLE 1. THE COMPOSITION OF THE BLOOD AFTER FOUR WEEKS OF TREATMENT WITH CORTISONE ACETATE

(All Results Are Given in Mean ± Standard Error)

(I) Component	(II) Control rooster	(III) Control hen	(IV) Control capon	(V) Cortisone rooster	(VI) Cortisone hen	(VII) Cortisone capon	(VIII) Cortisone +estrogen rooster	(IX) Cortisone +estrogen hen	(X) Cortisone +estrogen, capon
No. birds	10	10	2	10	15	10	4	12	2
Calcium, mg.%	9.4 ±1.0	19.4 ±6	10.1 ±0.9	15.0 ±3	14.1 ±2	16.1 ±6	99 ±19	116 ±12	94 ±8
UF Ca, mg.%	6.2 ±0.5	7.0 ±1	6.0 ±0.4	8.4 ±1.8	8.6 ±1.6	8.0 ±2.0	8.8 ±1.4	8.9 ±1.2	8.7 ±1.5
Inorganic phosphorus, mg.%	6.0 ±0.7	8.0 ±2	6.1 ±1	6.0 ±1.6	6.2 ±1.0	5.8 ±0.8	23.9 ±0.3	24 ±1.1	19.7 ±0.3
Total protein, gm.%	4.2 ±0.6	4.9 ±1.8	3.9 ±0.6	4.0 ±0.6	3.7 ±0.7	3.6 ±0.2	8.4 ±0.6	8.7 ±0.5	38.0 ±0.5
Albumin, mg.%	1.1 ±0.1	1.7 ±0.2	1.0 ±0.2	1.0 ±0.4	1.2 ±0.3	1.0 ±0.2	1.0 ±0.1	1.0 ±0.1	0.9 ±0.2
Globulin, mg.%	3.1 ±0.3	3.2 ±0.9	2.9 ±0.1	3.0 ±0.3	2.5 ±0.4	2.6 ±0.7	7.4 ±0.5	7.7 ±0.5	7.1 ±0.2
A/G ratio	0.35 ±0.04	0.51 ±0.5	0.31 ±0.02	0.33 ±0.3	0.48 ±0.4	0.37 ±0.9	0.13 ±0.1	0.11 ±0.1	0.12 ±0.1
Total lipid, mg.%	450 ±50	1500 ±505	875 ±25	900 ±100	1882 ±501	1200 ±205	12,120 ±900	15,191 ±370	16,320 ±309
Phospholipid, mg.%	155 ±32	402 ±349	155 ±5	320 ±51	268 ±54	245 ±7	3,001 ±382	4,364 ±944	4,460 ±694
Alkaline phosphatase*	9.2 ±1	12.1 ±2.9	11.1 ±0.8	12.0 ±1	13 ±3	13 ±4	12.0 ±3	14.1 ±2	9.0 ±8
Sugar, mg.%	208 ±8	229 ±19	220 ±17	210 ±11	237 ±41	211 ±55	239 ±41	286 ±5	168 ±28

* = King-Armstrong Units.

TABLE 2. THE EFFECT OF CORTISONE ON ADRENAL AND OVARIAN WEIGHTS, EGG PRODUCTION AND INTRAMEDULLARY OSTEOGENESIS

No. of birds	Sex	Treatment	Wt. of adrenals mg./kilo. (M ± E)†	Wt. of gonads gm./kilo. (M ± E)†	Average no. eggs per hen 8 weeks	Osteoporosis in cortex	Intramedullary bone
10	M	control	33.1 ± 4	10.0 ± 2	—	0	0
10	F	control	85.0 ± 3	18.8 ± 3	14–20	1–2+	1+
2	C	control	80.2 ± 7	—	—	2+	0
10	M	cortisone	20.1 ± 1	5.1 ± 1	—	2+	0
15	F	cortisone	19.4 ± 2	0.9 ± 0.1	2*	3–4+	3+
10	C	cortisone	10.9 ± 4	—	—	3–4+	0
4	M	cortisone & estrogen	24.9 ± 3	0.9 ± 0.1	—	1–2+	4+
4	F	cortisone & estrogen	9.6 ± 4	1.0 ± 0.1	7*	3–4+	4+
2	C	cortisone & estrogen	11.6 ± 6	—	—	3–4+	4+
5	M	estrogen	100.2 ± 10	2.7 ± 1	—	1–2+	4+
5	F	estrogen	111.1 ± 12	3.0 ± 1	10	2–3+	4+

† = Mean ± Standard error.
* = Miniature eggs.

for peak egg production are afflicted with a physiological form of osteoporosis (Figs. 2A and 3C).

Control capons. The levels of 11 constituents of the blood of two capons, shown in Table 1, Column IV, and the weight of the adrenals, shown in Table 2, resembled that of the rooster. The length of the average tibia was 10% greater; the cortex was also slightly thinner and more rarefied. The Haversian canals generally measured 20 to 80 μ in diameter and there were a few irregularly distributed abnormal large Haversian canals.

Cortisone-treated roosters. Ten birds were injected with cortisone acetate, 50 to 300 mg. per week for periods varying from 2 to 8 weeks. The gonads were reduced approximately 50% and the adrenals 30% when expressed in

TABLE 3. THE HEXOSAMINE AND COLLAGEN CONTENT OF VARIOUS FORMS OF BONE TISSUE AFTER FOUR WEEKS OF TREATMENT WITH LARGE DOSES OF CORTISONE

No. of birds	Sex	Treatment	Tissue	Hexosamine*	Collagen*
3	M	Control	Cortex	40.0 ± 9.1**	300 ± 47**
3	M	Control	Spongiosa	46.2 ± 6.4	250 ± 41
5	F	Control	Cortex	31.3 ± 3.7	280 ± 31
5	F	Control	Spongiosa	46.2 ± 4.1	161 ± 30
5	F	Control	Intramedullary bone	40.3 ± 3.8	128 ± 12
3	M	Cortisone-treated	Cortex	27.0 ± 3.8	323 ± 14
3	M	Cortisone-treated	Spongiosa	42.0 ± 3.1	282 ± 36
3	F	Cortisone-treated	Cortex	22.5 ± 2.0	500 ± 42
3	F	Cortisone-treated	Spongiosa	35.0 ± 2.4	116 ± 10
3	F	Cortisone-treated	Intramedullary bone	40.3 ± 4.0	128 ± 11
5	F	Estrogenized	Cortex	31.0 ± 3.2	363 ± 29
5	F	Estrogenized	Spongiosa	40.0 ± 3.6	215 ± 20
5	F	Estrogenized	Intramedullary bone	43.0 ± 6.7	128 ± 12

* = Micrograms per 100 grams of dry weight of tissue.
** Arithmetical average ± standard error.

FIG. 1. Microradiographs ×20 of the compact bone of the shaft of the tibia in cross-section; A., control rooster showing dense cortical bone; B., capon treated with 150 mg. of cortisone acetate (50 mg. per week for 3 weeks), showing beginning of enlargement of haversian canals; C., rooster treated with 2400 mg. of cortisone acetate (200 mg., 3×week for 4 weeks), showing a thin cortex with many enlarged haversian canals; D., rooster similarly treated with 2400 mg. of cortisone, following recovery for 6 weeks, showing repair of the cortical bone and overgrowth.

gm. or mg. per kilo. of body weight. The composition of the blood and bone of roosters with hypercortisonism, however, was the same as that of control roosters until the 4th week of treatment. After 4 weeks, many of the birds showed hypercalcemia, associated with elevation of the ultrafilterable fraction, and normal levels of plasma protein. A moderate, but definite, osteoporosis appeared after 6 weeks of cortisone injections. By 8 weeks, the hexosamine content of the cortical bone tissue had diminished slightly, while the content of collagen remained unaltered. Microscopically, however, there was no qualitative change in the staining properties of the bone tissue. There was no intramedullary new bone (Table 1, Column V) (Figs. 1C and 3B).

FIG. 2. Microradiographs ×5 of cross-section of the tibia including the marrow cavity, 3 cm. distal to the knee joint; A., control laying hen, showing cortex with many large haversian canals and lined with a spongy deposit of intramedullary bone; note that the structure of the compact bone of the normal laying hen resembles the structure of the cortex of the heavily cortisonized rooster; B., hen after laying was suppressed by 1200 mg. of cortisone (300 mg. per week for 4 weeks), showing highly osteoporotic cortex with large amounts of unabsorbed intramedullary bone.

Cortisone-treated capon. Ten birds were injected with cortisone acetate (Merck) in doses varying from 15 mg. to 50 mg. per kilo. per week for periods from 2 to 16 weeks.

The blood of cortisone-treated capons (Table 1, Column VII), showed hypercalcemia and slight hypoproteinemia. The hypercalcemia, under these circumstances, was due to an elevation of the ultra-filterable fraction; this differs from the hypercalcemia of the laying hen, which is due to an elevation in the nonultrafilterable fraction of the serum protein. There was no hyperglycemia. The weight of the adrenals in mg. per kilo. of body weight was reduced to 12% of that of control capons. The tibia was longer, lighter, and more porous in the untreated capon than in the rooster. In the capon, after 4 weeks of cortisone treatment, the cortex became highly

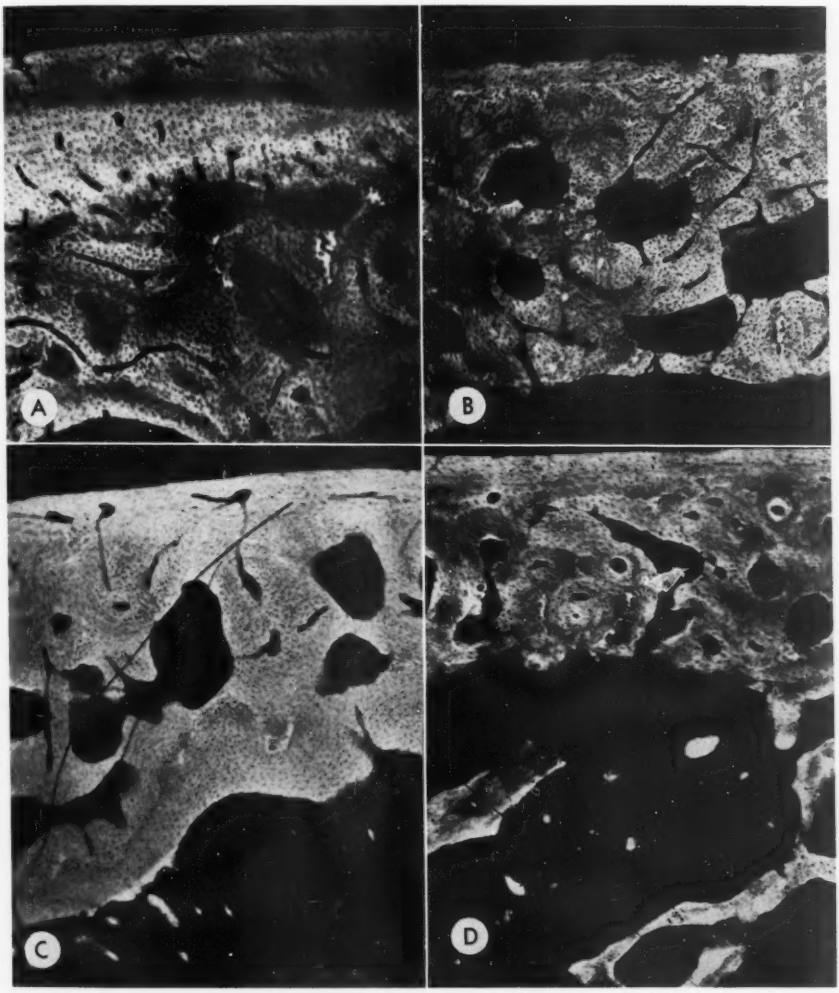

FIG. 3. Microradiographs ×50 to show osteones of the cortex of the tibia; A., control rooster, showing mainly lamellar bone with a few mature osteones in the deep layers and variable amounts of new bone with low density calcification; B., rooster after 4 weeks of treatment with 2400 mg. of cortisone showing enlarged vascular channels, unremodelled osteones (one osteone, upper left, shows a lamella of new bone); C., control hen, showing the many enlarged vascular channels similar to those found in the cortisone-treated osteoporotic male except that there are many osteones with lamellae of new bone; D., hen after four weeks of treatment with 1200 mg. of cortisone showing thin cortex, enlargement and distortion of many of the vascular channels, high density calcification (hypercalcification), and no osteones with low density lamellae of new bone.

rarefied by resorption of compact bone and enlargement of the Haversian canals, and there was severe osteoporosis. Osteoblasts and osteoclasts were scarce; the connective tissue covering the trabeculae contained fewer cells and more fluid spaces than normal. There was no intramedullary new bone (Fig. 1B).

Cortisone-treated laying hens. Fifteen birds were injected with cortisone acetate, 50 to 300 mg. per week for periods varying from 2 to 8 weeks. The levels of the plasma protein, lipid, and phospholipid were lower (Table 1, Volumn VI) than found in control laying hens (Table 1, Column III). The The levels of calcium were the same as in controls during the first week of treatment. After the first week, the hens ceased to lay and the level of the total calcium fell to the same as that found in cortisone-treated capons, roosters, and resting hens. After the second week, however, the level of the ultrafilterable fraction of the serum calcium was markedly elevated. Paper electrophoresis showed that there was neither phosphoprotein or X_1 fraction nor phospholipid-lipoglycoprotein or X_2 fraction (17) that is always associated with the hypercalcemia of normal laying hens. The excess calcium in the serum from the cortisone-treated hen was found in the ultrafilterable fraction and could not have been bound to phosphoprotein. The level of the serum albumin was relatively normal but the globulin was generally low. There was no hyperglycemia, either in the laying or the resting hens.

The ovaries, measured in gm. per kilo. of body weight, after 8 weeks of treatment with cortisone, were reduced to 4 per cent of those of the control hens. The number of follicles appeared to be the same as normal but there were none that were growing in size, yellow in color, or storing egg yolk. The weight of the adrenal glands, measured in mg. per kilo. of body weight, after 8 weeks of treatment with cortisone (Table 2), were reduced to 22 per cent of the weight of those of control hens.

The cortex of the long bones was markedly reduced in the thickness and showed enlargement of the Haversian canals with a scalloped endosteal surface. Remains of intramedullary bone were found, as in laying hens, as long as after 4 weeks of injections of cortisone; the trabeculae in these areas were thin and sometimes incompletely calcified. The skeleton, as a whole, was hard, but light, in weight, extremely brittle, and difficult to cut because of excessive fragmentation. Microscopically, the compact bone was strongly eosinophilic; the intramedullary bone was basophilic and stained metachromatically with toluidine blue or azure, and positive with periodic acid-Schiff reagents. The cortex was fully calcified but the spongiosa was partially uncalcified and markedly attenuated. Wilder's stain for collagen showed normal fiber bundle and cement lines (Figs. 2B and 3D).

After 4 weeks of treatment with large doses of cortisone, the hexosamine content of the osteoporotic skeleton of the hen was as low as 22.5 μg. per

100 gm. of the dry weight of the cortical bone; this is almost 30% less than in normal birds. The spongiosa from the metaphyses was less affected. The hexosamine content of the intramedullary bone deposits was hardly affected, suggesting that it consisted largely of unresorbed bone. The spongiosa and cortical bone were resorbed and may have been replaced, to a limited extent, during the period of cortisone treatment; in the process, the bone tissue lost hexosamine containing components in some unknown way. The collagen content was higher in cortical bone than spongiosa or intramedullary bone, but generally unaffected even by prolonged treatment with very large doses of cortisone (Table 3). The standard error in the results of these methods suggests that the changes in hexosamine and collagen content of the tissues were relatively slight even in animals at an advanced stage of adrenal hypercorticoidism.

Egg production. Laying ceased in hens (10 to 16 months of age) at the end of the first week of cortisone treatment. Several very young birds, however, persisted in laying for 2 to 4 weeks during treatment even with very large doses. These laid only one or two dwarf eggs having a weight of 10.3 to 15.9 gm. or less than half that of a normal egg. Such anomalous eggs were shell-less and some showed a stalk or membranous attachment (Table 2, Fig. 4).

Cortisone- and estrogen-treated rooster. Four roosters were treated with simultaneous injections of 125 mg. of estrogenic substances, Premarin (Ayerst), and 300 mg. of cortisone acetate (Merck), per week, as described above, and the effects on blood and bone were entirely similar to those observed in capon. The testes showed reduction of weight, to 10% of normal controls, as compared with only 50% reduction in roosters treated with cortisone alone. The depressant effect of estrogen may be attributed to inhibition of pituitary gonadotropin (Table 1, Column VIII); (Table 2).

Cortisone- and estrogen-treated hen. Twelve hens were also treated with simultaneous injections of estrogen and cortisone for 2 to 8 weeks. The changes in the composition of blood produced by estrogen were not prevented by cortisone and were the same in magnitude as in control hens (Table 1, Column IX). The exogenous estrogen, however, did not restore the progress of synthesis of yolk protein in the ovarian follicles and decreased, rather than increased, the weight of the ovary. The adrenal glands, measured in mg. per kilo. of body weight, reduced to approximately 5% of those of normal control hens.

Cortisone- and estrogen-treated capon. Two capons were treated with simultaneous injections of estrogenic substances, Premarin, 125 mg. per week, for 2 weeks, and cortisone acetate, 15 to 50 mg. per week for 2 to 10 weeks, and the blood showed hypercalcemia, hyperproteinemia, and lipema as extreme as in the intact animal. Cortisone did not suppress the production of yolk proteins or lipids produced by the liver under the stimulus of estro-

gen (17–18). The bones showed extreme osteoporosis. There were small, irregular deposits of intramedullary new bone.

The long bones showed as much intramedullary osteogenesis as seen in controls, but some of the new bone was abnormal. The trabeculae were thin and composed of large amounts of incompletely calcified matrix or osteoid tissue without any calcification. Estrogen did not prevent the development of osteoporosis in the cortex (Table 1, Column X).

Recovery from Osteoporosis. Six roosters were injected with 100 mg. of cortisone acetate 3 times per week for 8 weeks, and sacrificed at intervals of 1,2,3,4,6, and 8 weeks after the last injection. During the first 2 weeks, the levels of the serum total protein rose from below to above the normal and gradually returned to the level of the average control. The level of calcium remained elevated as high as 15 mg. per cent as long as 6 weeks after cortisone was discontinued. The levels of other components varied irregularly. The weights of the adrenals and testes also varied irregularly and were ap-

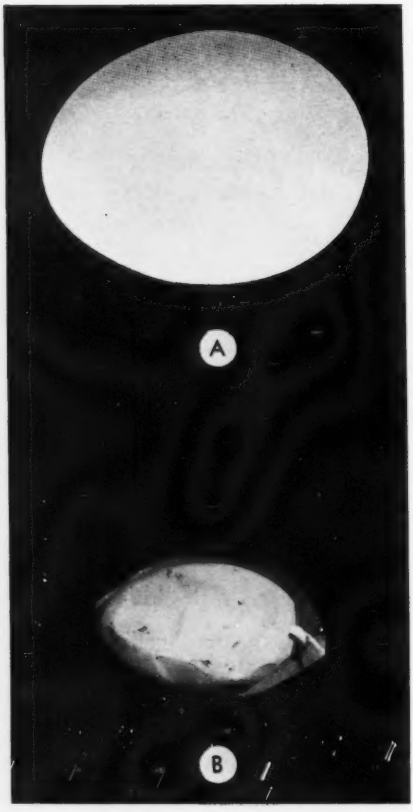

FIG. 4. Photographs of eggs from a pullet treated with cortisone, 300 mg. per week; A., normal egg laid during the first week of treatment; B., dwarf egg with stalk laid during the third week.

proximately 10% below the normal weight in one bird sacrificed after 8 weeks of recovery.

The bones showed an interesting rebound phenomenon in which the cortical bone became denser than normal and sealed from the marrow cavity by a thick layer of endosteal new bone. The sharp line of cement substance demarcated the old and the new compact bone. The enlarged Haversian canals of the osteoporotic compacta became filled in with new bone gradually over a period of 4 to 6 weeks (Fig. 1D).

DISCUSSION[2]

Osteoporosis, resulting from pharmacodynamic doses of cortisone, occurred in roosters and hens when definite alterations were present in the composition of the blood, the size and weight of adrenals and gonads, and the structure of the skeleton. The adrenals and gonads were markedly atrophied owing presumably to direct effects, as well as indirect effects upon the pituitary secretion of ACTH and gonadotropic hormones. Hypoproteinemia and hypercalcemia appeared in the blood. Osteoblastic activity ceased and bone tissue was resorbed without the appearance of osteoclasts.

Adrenal cortex. In investigations, to be published later, we found that culls of White Leghorn flocks, afflicted with spontaneous osteoporosis, had plasma levels of 7 to 9 μg. of corticosterone and 2 to 3 μg. of 17-hydroxycorticosterone per 100 cc. of blood plasma. Corticosterone is the chief glucocorticoid in the blood of avian species (23). Quantitative determination of the various cortical hormones excreted daily in the urine and feces in normal, osteoporotic, and ACTH or cortisone-treated birds, should provide additional valuable information about adrenal physiology.

Gonads. The levels of estrogen, androgen, 17-ketosteroids in blood and excreta should also be examined by newer methods in birds with osteoporosis, either spontaneous or induced by cortisone treatment.

Hypercalcemia. The level of calcium in the serum of the rooster was remarkably constant, 9.4 ± 1.0 mg.%. Following cortisone treatment, roosters developed a hypercalcemia associated with an elevation of the ultrafilterable fraction of the serum calcium. The mechanism of this change is not

[2] The terms anabolism, antianabolism, and catabolism were avoided deliberately in this report for the following reasons: estrogens in large doses are powerful growth-inhibitors and are antianabolic for epiphyseal growth, but in birds are anabolic for intramedullay bone; androgens are anabolic for somatic growth, but are antianabolic for intramedullary bone; cortisone is antianabolic in small doses and catabolic in large doses, and has much less effect on intramedullary bone than on somatic growth. While these terms are useful for clinical metabolic balance studies, according to Lipmann (22), they will disappear with further progress and understanding of the chemistry of metabolism.

clear. One possibility is that the high calcium content of commercial diets for domestic fowl, fed to a bird in which bone formation and kidney function were suppressed by cortisone, causes the level of calcium in the plasma to rise. Grollman (24) found hypercalcemia following cortisone treatment of nephrectomized dogs.

The laying hen differs from the rooster and the capon in that she, normally, has a hypercalcemia. The calcium that is added to the plasma, however, is chiefly nonultrafilterable and bound to a phosphoprotein that is formed along with a phospholipid-lipoglycoprotein, and is associated with hyperglycemia, lipemia, and other changes related to transport of special substances for the growing ova. Such changes normally found in the blood of the laying hen can be produced in the rooster simply by injections of estrogen. None of these changes can be induced in the rooster, capon, or hen, by cortisone.

Cortisone treatment in the hen and capon produced a slight lowering of the level of the serum globulin with no appreciable effect upon the level of albumin; it did not produce a corresponding hypoproteinemia in the rooster. The levels of phosphoprotein, total protein, phospholipid, and phosphorus in transport in the plasma of the hen, measured either by chemical methods or by paper electrophoresis, were markedly suppressed by cortisone. Cortisone, in suppressing secretion of gonadotropin (26) and normal ovarian function, eventually interrupted the production and utilization of these proteins. The liver (the organ that synthesizes X_1-X_2), however, was relatively unaffected because birds with severe hypercortisonism, injected with large doses of exogenous estrogen, were able to produce the same degree of phosphoproteinemia, lipemia, and hypercalcemia as normal birds. Thus, cortisone would appear to exert a greater adverse influence upon the function of the pituitary, adrenal cortex and the gonadal organs that upon other tissues. It is likely, however, that competition for protein transport mechanisms may function to favor estrogen, rather than glucocorticoid hormones in birds, as in mammals, as postulated by Szego (25).

Avian osteoporosis. The cortex of the long bones of the laying hen is normally thinner, and much more porous than in the rooster, and suggests that a low grade form of osteoporosis is physiological in the female during and after a strenuous period of reproduction. Meister (27) described osteoporosis in Canadian geese during the molt and ascribed it to endocrine influences. The molt is generally regarded as under the control of the thyroid, but the effects of other endocrine organs and the relationship to osteoporosis are not clear. Cortisone, low calcium diet, parathyroidectomy, high temperatures, and many other conditions, can precipitate the molt. The characteristic effect of cortisone upon the skeleton was to accentuate the normal state of osteoporosis of the hen, promote the condition in the capon, and initiate the disorder in the rooster. The mechanism of the bone-resorb-

ing action of cortisone was obscure. Injections of this steroid caused the cortex to become thin and rarefied, the Haversian canals to enlarge, the endosteal surface of the bone to develop a scalloped outline (as from erosion progressing from the inside to the periosteal surface), and each bone to lose approximately 30% of its osseous mass. This occurred with hardly any cellular action of osteoclasts. The bone tissue, prior to its resorption, by some elusive change, may have lost hexosamine without reduction in its content of collagen. The concentration of hexosamine reflects the amount of mucoprotein and other components of the labile fraction of the body protein stores. Cortisone is generally believed to mobilize only the labile protein while it inhibits the synthesis of collagen and other components of the stable protein stores.

The chemical change in the bone tissue, concomitant with a gradual disappearance of both osteoblasts and osteoclasts, was most extreme in cortical bone, moderately advanced in the spongiosa, but hardly recognizable in intramedullary deposits. The intramedullary bone is formed under the influence of estrogen and appears to be resorbed when the level of estrogen in the blood declines. It is interesting to compare the effects of cortisone and parathyroid hormone on this type of bone tissue. Parathyroid hormone, which invokes osteoclastic activity, detaches intramedullary bone from cortex; cortisone produces resorption without formation of osteoclasts and does not detach intramedullary bone from the cortex. This suggests that the two hormones exert their effects on bone tissue by different mechanisms.

Spontaneous fractures have been described in birds following treatment with estrogen (28–29). This has been attributed to osteoporosis (19). One possibility is that the causative factor is hypersecretion of glucocorticoids through stimulation of the adrenocorticotropin production by the pituitary. This requires determination of the adrenal cortical steroids excreted in urine of these birds. Androgenic steroid hormones have been administered to hens in our laboratory and found to protect the skeleton against osteoporosis and the results will be presented in a later communication.

Acknowledgment

Cortisone acetate used in above experiments was generously supplied by Dr. Nicholas E. Capeci, Merck, Sharp, and Dohme Research Laboratories. The estrogenic substances were supplied by Dr. John J. Jewell, Medical Director, Ayerst Laboratories, Inc.

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STEROIDOGENIC ACTIVITIES OF PURIFIED α, β, γ, δ-CORTICOTROPINS AND PEPSIN DEGRADATION PRODUCTS OF β-CORTICOTROPIN[1]

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ABSTRACT

The adrenocorticotropic activity of the purified corticotropins α_1, α_3, α_4, β, γ_1, γ_2, δ_1 has been determined vs. the U.S.P. Reference Standard for corticotropin in an *in vitro* assay based on corticoidogenesis. The potencies of these various preparations in U.S.P. U./mg. were: $\alpha_1 = 11.2 \pm 2.1$; $\alpha_3 = 34.0 \pm 2.8$; $\alpha_4 = 25.8 \pm 5.1$; $\beta = 94.5 \pm 10.6$; $\gamma_1 = 11.6 \pm 2.0$; $\gamma_2 = 21.7 \pm 4.1$; $\delta_1 = 27.6 \pm 6.9$. The pepsin degradation products of β-corticotropin, $P_{2,3,4}$ had activities respectively of 27.5 ± 7.9; 31.4 ± 4.7; 21.5 ± 10.0, in U.S.P. U./mg. All these preparations had been reported previously to have equal and maximal activity (80–100 U./mg.) in the adrenal ascorbic acid depletion test.

SHEPHERD *et al.* (1, 3) and Bell *et al.* (2) have reported previously in their "Studies with Corticotropin" that the specific activities (i.e number of U.S.P. units for corticotropin/mg.) of all their highly purified ACTH-peptides[2] α_1, α_2, α_3, α_4, β, γ_1, γ_2, γ_3 were identical and *maximal* (80–100 U./mg.) in the classical adrenal ascorbic acid (A.A.A.) depletion test (4). Fraction δ_1 was the only one reported with a lower specific activity (10 U./mg.); however, the material labelled δ_1 was known to be non-homogeneous. The yields of the various components after counter current fractionation of oxycellulose-ACTH (OC-ACTH) were respectively: 12% α, 49% β, 19% γ and 20% δ. Since the main purified fractions showed equal and maximal adrenocorticotropic activity, Shepherd, Bell *et al.* decided that "the β-fraction (would be) selected for further characterization because it represented the largest single active component (ca. 50%) of OC-ACTH" and for other secondary reasons related to the number of degradation products by pepsin.

The series of experiments reported here was undertaken with the hypothesis that with a test based on corticoidogenesis and not the ascorbic acid depletion, one might find that one of the corticotropins had a greater specific activity than the others, thus qualifying itself to represent the

Received September 25, 1959.

[1] This work was supported by a grant (No. A-1276) from the National Institute for Arthritis and Metabolic Diseases, U. S. Public Health Service and Contracts No. AF 41(657)-17 and AF 41(657)-224 with the School of Aviation Medicine, U. S. Air Force, Randolph Field, Texas.

[2] The terminology used in this paper is that of Bell *et al.* (2).

main product endowed with the maximal biological activity. It has been decided to report these data in view of the renewed interest in the "minimal peptide sequence" of natural or synthetic origin endowed with adrenocorticotropic activity (5, 6) and also because of the increasing evidence that the classical ascorbic acid depletion test may not consistently parallel true corticoidogenesis as measured *in vivo* under endogenous or exogenous corticotropic stimulation (7, 8, 9, 10, 11).

MATERIALS AND METHODS

Aliquots of the following purified materials (of hog origin) were received in powder form from Dr. P. H. Bell, Lederle Laboratories Division, American Cyanamid Co., New York: α_1, (S-413-182); α_3, (S-1079-28); α_4, (S-1079-28); β, (S-776-98); γ_1, (S-776-35); γ_2, (S-776-35); δ_1, (S-1079-24); P_2, (S-1892-34); P_3, (S-1692-116); P_4, (S-1692-116). Reference is made to the papers (1, 2, 3) by Shepherd *et al.* and Bell *et al.*, for description of these various preparations. The samples were received in this laboratory in June 1957 and assays were run, for the corticotropins between August 12–23, 1957, for the pepsin degradation products between September 20–24, 1958. In both laboratories the materials were always kept refrigerated and in desiccators. According to Dr. P. H. Bell, the α, γ, δ samples were 85–90% pure; the β sample was of the highest degree of purity and had been used for the degradation work (2, 3). These substances were studied for their adrenocorticotropic activity in the *in vitro* assay of Saffran and Schally (12). In all experiments the methylene chloride extracts of the adrenal incubation fluids were chromatographed on paper in the E2B system of Eberlein (14) with corticosterone and aldosterone reference standards. The corticosterone area was eluted and the concentration of the steroid in the eluate measured by U.V. absorption in the Beckman spectrophotometer; the aldosterone area was eluted and Molenaar's modification (16) of the Gornall reaction was carried out on the eluate for quantitative measurement in the spectrophotometer (studies in collaboration with Drs. G. W. Clayton and J. D. Smith). In several instances the eluate of the aldosterone area was assayed in the adrenalectomized rat for Na-retaining activity against l- or dl-aldosterone (studies in collaboration with Dr. H. S. Lipscomb). The U.S.P. Reference Standard for corticotropin was used throughout the series of experiments. Samples were dissolved in 0.01N HCl and kept frozen until assay, usually 1 to 5 days later; the U.S.P. Reference Standard was treated similarly and simultaneously. Assays were of the classical programming (4 points, in duplicate) with S_1, U_1/S_2, $U_2=\frac{1}{3}$. All assays were studied by analysis of variance and factorial analysis (13) with variance ratios calculated for significance of sample, slope and divergence at $p=0.05$. Confidence limits (antilog $C^2M \pm Cts_M$) of the computed potency and the index of precision $\lambda=s/b$ were calculated for each experiment (Table 1). When several assays were run on a particular substance, the mean potency reported was computed from the average of the unweighted log potencies. The standard error (S.E.) of mean potencies as reported in Table 1, has been computed from the average of the *unweighted* and *crude* s_M of each assay, thus placing equal weight on the *internal* (within assays) and *external* (between assays) variances of the combined potency estimates.

RESULTS

The specific activity in U.S.P. U./mg. of the various corticotropins $\alpha_{1,3,4}$, β, $\gamma_{1,2}$, δ_1 and the pepsin-degradation products $P_{2,3,4}$ of β-corticotropin are reported in Table 1. Results of the classical assays were consistent with

TABLE 1. STEROIDOGENIC ACTIVITIES OF PURIFIED ADRENOCORTICOTROPINS AND OF PEPSIN DEGRADATION PRODUCTS OF β-ACTH

Substance tested	Specific activity in USP U./mg. with confidence limits* of assays at P = 0.05				mean potency in USP U./mg. ± S.E.**	$\lambda = s/b_c$ of each assay
α_1	13.8 (2 – 25)	12.5 (4 – 21)	14.4 (10– 26)	6.2 (4–9)	11.2± 2.1	.125 .086 .184 .066
α_2	37.7 (21 – 60)	33.4 (18 – 25)	31.4 (13– 53)		34.0± 2.8	.101 .099 .122
α_4	29.9 (6 – 51)	22.3 (5 – 38)			25.8± 5.1	.145 .124
β	91.9 (57 –195)	90.0 (22 –254)	102.0 (57–372)		94.5±10.6	.106 .231 .139
γ_1	11.1 (4 – 33)	12.2 (0.6– 18)			11.6± 2.0	.121 .162
γ_2	26.7 (18 – 44)	17.6 (6 – 48)			21.7± 4.1	.079 .149
δ_1	21.9 (1.3– 34)	34.8 (13 – 64)			27.6± 6.9	.181 .134
β-P_2	27.5 (12 – 61)				27.5± 7.9	.130
β-P_3	31.8 (11 – 92)	31.0 (11 – 87)			31.4± 4.7	.215 .182
β-P_4	21.5 (1 – 31)				21.5±10.0	.188

* Confidence limits computed by $C^2M \pm Cts_M$.

** S.E.: Standard error of the mean potency M computed by averaging unweighted s_M and crude s_M for each series of assays (see text). For β-P_2 and β-P_4, S.E. of potency calculated as: (100 (antilog s_M) – 100) ×antilog M.

data obtained from the quantitative measurement of the corticosterone in the adrenal incubation fluids. Results obtained with the procedures aimed at studying aldosterone release were inconsistent because of the exceedingly small amounts of substance found, whenever any was detected; this should be related probably to the small amounts of adrenal tissue involved (16) and the relatively short incubation time utilized here.

The various corticotropin preparations differ widely in their specific activities in the steroidogenic assay in contradistinction to their similar potencies at 80–100 U./mg. in the A.A.A. depletion test as reported by Shepherd *et al.* (1, 3) and Bell *et al.* (2). β-corticotropin, the most potent substance in the experiments reported here, is the only one for which the specific activity corresponds to the value obtained earlier in the A.A.A. depletion test (1). Whereas $\alpha_{1,3,4}$, $\gamma_{1,2}$-corticotropins appear less potent in the steroidogenic test than in the ascorbic acid depletion method, δ_1 conversely is more potent here (27.6 ± 6.9 U./mg. vs. 10 U./mg. in A.A.A.). δ_1 is not a homogeneous material and is known to be contaminated with melanocyte stimulating hormone (MSH). Its considerable corticotropic activity cannot be attributed to the MSH: recent studies (5) have indicated that in the assay system used here highly purified β-MSH has no corticotropic activity and α-MSH has only a minimal, though not negligible, activity (ca. 3U./mg.). The pepsin degradation products of β, $P_{2,3,4}$ reported as active as the "starting β-corticotropin" by the A.A.A. method (2, 3) are much less

potent here than the intact 39-peptide β. If α_4 is truly esterified-β as suggested by Shepherd *et al.* (1), the process had led to considerable loss of activity (Table 1).

DISCUSSION

Possible physiological significance of the absence of correlation between the data observed here with the corticoidogenic assay and those reported previously with the adrenal ascorbic acid depletion assay (1, 2) is only speculative. One can only regret that with the chemical studies on corticotropin by Bell *et al.* (2) and Shepherd *et al.* (1, 3) no rigorous quantitation of the biological activities of these unique materials was even considered (1). In view, however, of the usually excellent slope of the ascorbic acid depletion assay and also in view of its short range of optimal utilization, it would be very surprising that one could err by factors of 3 to 10 in determining in successive assays the potency of an unknown, thus accounting for the discrepancies of specific activity in the two assay systems. Against this proposed explanation is also the remarkable coincidence of the potencies for β-corticotropin by the two assay methods. One might consider the possibility that some samples may have deteriorated even though kept in the best conditions in both laboratories; the correspondence in the two assay methods of the specific activities for β-corticotropin and also the *greater* potency for δ_1 found here as compared with the A.A.A. depletion test are not in agreement with the idea of inactivation. One may envision that the ascorbic acid depletion test does not differentiate between closely related molecules (since with it, all the corticotropins had equal and maximal potency) whereas the assay based on corticoidogenesis shows these subtle differences. Another formulation of the same concept would be to hypothesize that molecules of corticotropins differing only in minor aspects of their structures may have different affinities for the "ACTH receptors" of the adrenocortical tissue, thus accounting for variable steroidogenic activation. The *in vitro* technique with its long period of contact with the adrenal tissue and its virtual absence of extraneous inactivation of ACTH would be an ideal situation for observing such an eventuality. This has been recently discussed in relation to species specificity of the adrenocorticotropins (17, 18). The hypothesis that the α (with the possible exception of α_4), γ and δ materials might be unfinished products in the biosynthesis of the final hormone, β, is of interest but impossible to study at the time for limitations of knowledge on this particular aspect of endocrinology.

The more objective aspect of this data has been to confirm that β-corticotropin is indeed the most potent corticotropic material of the series α through δ. The choice of Shepherd *et al.* (1) and Bell *et al.* (2) of the β-fraction on the basis of its per cent weight of the starting material is thus reinforced by the findings on its specific activity in the steroidogenic assay as compared to that of the other peptides, information that the ascorbic acid

depletion test had not evidenced. The lack of correlation between the data of the steroidogenic assay and those of the ascorbic depletion test with all the cautions set forth above for their acceptance may be related to similar occurrences observed with endogenous (8, 9, 10) or exogenous (7, 11) stimulation of the adrenal cortex.

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MENSTRUAL IRREGULARITIES IN RHESUS MONKEYS ELICITED BY RESERPINE ADMINISTERED ON SELECTED DAYS OF THE CYCLE[1]

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ABSTRACT

Female rhesus monkeys were treated with reserpine during the proliferative phase of the menstrual cycle, in varying dosages and patterns of administration. One mg. per kg. of body weight given on three successive days, tended to prolong the menstrual cycles, especially when injections were begun on day 6 or before. When reserpine was given on days 2 to 7, 1 mg. per kg. per day prolonged all cycles. Half this dose prolonged 60% of the cycles. The larger dose given from days 7 to 12 prolonged only 25% of the cycles. The larger dose prolonged all cycles when the drug was given from days 2 to 12, but 0.2 mg. per kg. prolonged only about 16% of the cycles. Some monkeys were more susceptible to reserpine than others. As judged by the criteria of (a) a decrease in the amount of sedimentation observed in vaginal lavages, (b) a reduction in the number of cornified cells and (c) an increase in the number of leucocytes in vaginal smears, reserpine gives evidence of causing a temporary suppression of circulating estrogen.

REPRODUCTIVE function in some species of animals is modified by the administration of reserpine. Gaunt *et al.* (1) found in rats an alteration in the estrous cycle and a reduction in fertility. Barraclough (2) inhibited ovulation in the same species. In rhesus monkeys, reserpine given daily in a dose of 1 mg. per kg. prevents occurrence of menstruation for as long as 100 days. Upon withdrawal of the drug, cycles are promptly renewed. Judged by the criterion of cellular desquamation in the vaginal lavage as well as by laparotomy, ovulation is suppressed during the period that reserpine is given (3).

Everett and Sawyer (4) found that the rat ovulates at a given hour in the night and that this may be blocked by anesthesia administered at a given hour in the afternoon of the same day. It became of interest to determine whether reserpine, given in selected doses and on selected days of the menstrual cycle, could affect the duration of the cycle. This was the purpose of the experiment reported in the present paper.

Received October 30, 1959.

[1] Supported by grants from the Population Council, New York City and the U.S.P.H.S. Grant RG 4728.

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METHODS AND MATERIALS

Adult rhesus monkeys were obtained from a commercial source. The ages of the monkeys could not be specified and their maturity could not be judged because their condition upon arrival in the laboratory was usually very poor.

In the absence of good indices of maturity in the rhesus monkey, estimates of the animals' ages were derived from a consideration of their weight and menstrual regularity. After adequate nutrition for 3 months, the weights of the animals were within the norm of mature animals (4500 gm.). After one to three more months, menstrual phenomena occurred with increasing regularity. Relative menstrual regularity is itself an index of reproductive maturity in the rhesus monkey (5), and as may be noted in Table 1, by this criterion most of the animals were mature and fit for our experimental purpose.

Eleven monkeys became acclimatized to the conditions of the laboratory and formed the basis for this study. During the winter season of 1957–1958, three more animals with known menstrual records were added to the colony. By this time sufficient menstrual

TABLE 1. SUMMARY OF MENSTRUAL HISTORIES

Animal	No. of winter seasons studied	Summer menstrual cycles (days)	Number of menstrual cycles recorded		Mean control menstrual cycle length	St. Dev.
			Summer and/or experimental	Winter "control" "n"		
IV	3	regular	8	20*	29.5	2.01
V	2	irregular range: 25–61	9	8	30.5	10.24
VI	3	irregular range: 25–38	11	11	28.3	3.83
VII	2	irregular range: 27–47	6	8	26.8	4.68
IX	2	regular	4	12*	28.1	2.39
X	3	irregular range: 10–51	14	13	28.3	2.83
XI	2	irregular range: 32–85	3	4	36.2	11.85
XII	3	irregular range: 12–37	13	13	27.9	3.63
XIII	3	irregular range: 18–49	11	11	28.8	3.22
XIV	2	regular	9	9*	28.9	1.06
XV	1	—	1	2	33.0	2.88
XVI	2	regular	1	9*	26.5	2.13
XVIII	2	regular	1	7*	27.4	2.65
XIX	3	irregular range: 11–64	14	12	26.1	1.73

Total number of control winter cycles 121.
* Indicates summer menstrual cycles are included.

histories were available on the 11 original animals to make possible an estimate of mean cycle length in each case. Of the thirteen animals studied during the 1957–1958 winter season, seven have remained under study to date (1958–1959). Thus a menstrual history is available in these seven animals through three consecutive winter seasons.

It is known that there is a seasonal variation in menstrual regularity in the rhesus monkey (5) and that the summer cycles may be anovulatory (5). Approximately half of the animals of our colony demonstrated summer menstrual irregularity and these cycles are not included in estimating the control menstrual cycle data. Moreover, reserpine was given only in the winter and spring of the year in order to be certain that any drug-induced variation in cycle length would not be superimposed upon spontaneous summer variability.

During 1956–1957, cyclic activity did not become established until late winter, and

TABLE 2. RESERPINE GIVEN ON SELECTED DAYS OF MENSTRUAL CYCLE
(*Italicized* = cycles greater than two standard deviations from the controls.)

Monkey	Spring, 1957						1957–1958				
	1 mg./kg.						1.0	0.5	1.0	0.2	1.0
	3, 4, 5	4, 5, 6	6, 7, 8	7, 8, 9	8, 9, 10	9, 10, 11	2–7	2–7	7–12	2–12	2–12
IV	31						*48*				*49*
V		33	33					45	35	30	
VI	33					33			*50*	23	
VII			*47*				*48*			34	
IX				*72*			*89*	*41*			*46*
X	*89*			28			*40*	*36*			*49*
XI		19		32							44
XII	28							*40*	26	28	
XIII	*56*				*39*					*116*	
XIV										31	
XV	*40*										
XVI							*48*				
XVIII	*38*										*45*
	45										
XIX			*34*	23				28	26		*45*
Per cent cycles affected	62.5		66	25			100	60	25	16.6	83.3

in the case of one animal (XI), not until the 1957–1958 season (Table 1). Consequently, a limited number of cycles were available for study at that time.

During drug administration, the monkeys were observed six days a week. Vaginal smears from the posterior fornix were done by a carefully controlled technique and differentially stained by the Shorr method (see 6, 7). Vaginal lavage sedimentation tests (Hartman Index, 5) were done three times a week. Since observations in the absence of drug administration were made three days a week, the occurrence of well established uterine bleeding was assumed to be on the second day of the menstrual cycle; bleeding probably began on the previous day. If the lavage was only lightly tinged with blood, it was assumed that the day of observation coincided with the day of onset of menstruation.

A dose of 1 mg. of reserpine per kg. of body weight was given on each of three successive days of the menstrual cycle in the 1957 season. The sequences were days: 3, 4, 5; 4, 5, 6; 6, 7, 8; 7, 8, 9; 8, 9, 10; 9, 10, 11. The drug was given in a total of nineteen cycles, distributed as shown in Table 2. The following winter, reserpine studies were begun again after the irregularities in mensturation of the summer had subsided. During the winter of 1957–1958, reserpine was given during a total of twenty-six cycles, distributed as shown in Table 2. Daily doses of 0.5 and 1.0 mg. per kg. of body weight were given from Days 2 to 7 of the cycle. One mg. per kg. was given from Days 7 to 12. Doses of 0.2 and 1.0 mg. per kg. were given from Days 2 to 12.

The above patterns and dosages were designed to impose any effect the drug might

have on the post-menstrual part (proliferative phase) of the cycle (a) over a very short period of time; (b) over the first half of the pre-ovulatory phase; (c) over the last half of the pre-voulatory phase; and (d) over approximately the first half of the cycle.

RESULTS

Reserpine over three-day periods

One mg. of reserpine per kilogram of body weight given for three successive days as described under Methods, above, beginning from the third through the ninth days prolonged some cycles in seven of twelve monkeys (see Table 2). The criterion of an effect was that the cycle during which the drug was given should deviate by more than two standard deviations from the mean control cycle length. On this basis, the results are significant at the 95% confidence limit.

With treatment on Days 3, 4, and 5, five of eight cycles were lengthened. When reserpine was given on Days 4, 5, and 6, two cycles were not significantly different from the controls, but when administered on Days 6, 7, and 8, two of three cycles and on Days 7, 8, and 9, one of four cycles were increased in length. One cycle during which treatment was on Days 8, 9, and 10, was significantly longer than the control cycle and in one monkey treated on Days 9, 10, and 11, the cycle was unaffected.

Taken as a whole, this pattern of injection showed no clear predilection for any three day period of administration although treatment during the earliest part of the cycle tended to be most effective. At most, when reserpine was given during several cycles as just described, 25 to 75% of the cycles were prolonged. In this and other groups of experiments to be described below, the number of samples in each class of observations is not sufficient to permit application of the Chi square test. The results must be considered collectively, paying attention to the trends exhibited by the several groups of data. Cost, time and facilities barred amplification of the program.

Reserpine for six days

During the second winter, reserpine was given for longer periods of time, namely, in five doses over six days (Days 2 to 7; Days 7 to 12) and in ten doses over eleven days (Days 2 to 12). No injections were made on Sundays, hence the day of no injection in any series varied irregularly. The doses were as shown in Table 2.

When 1 mg. per kg. of reserpine was given, both the number and percentage of affected cycles were greater than in the three-day injection periods described above. One-half mg. per kg. given from Days 2 to 7 significantly lengthened the menstrual cycle in three of five cycles, with confidence at the 95% level. All of six cycles in which 1.0 mg. per kg. was given were significantly lengthened. However, 1.0 mg. per kg. of reserpine given

daily from Days 7 to 12 affected only one of four cycles. Reserpine injected for the earlier 6 day period is more effective, therefore, than when administered from Days 7 to 12.

Reserpine for eleven days

The larger dose of 1 mg. per kg. of reserpine was effective in prolonging five out of six cycles when given from Days 2 to 12, whereas 0.2 mg. per kg. affected only one of six cycles. The one monkey (XI) on the larger dose that failed to respond had highly irregular cycles and may have been a poor subject to begin with (see Table 1). In any case, the most effective ways to alter the duration of the menstrual cycle, of all the patterns and dosages employed, is to use (a) 1.0 mg. per kg. from Days 2 to 7, (b) from Days 2 to 12 or (c) on Days 3, 4 and 5.

Dosage of reserpine

When the frequency of affected cycles in the 1957–1958 series is considered from the standpoint of the dosage alone, irrespective of the days on which the drug is given, differences are seen between the three dose levels. Two tenths mg. per kg. daily affected one, or 16.6% of six treated cycles; 0.5 mg. per kg. affected three, or 50% of six treated cycles; and 1.0 mg. affected twelve, or 75.0% of sixteen treated cycles. The dose and timing, therefore, are both important factors which affect the duration of the menstrual cycle.

The question may be asked, if, when a cycle is prolonged by reserpine, is the prolongation of the cycle proportional to the dosage given? The mean values of the cycle lengths in all instances where they were prolonged differed from the mean lengths of the control cycles as follows: 11.6, 10.9, and 15.5 days for 0.2, 0.5 and 1.0 mgms. The differences from the means appear not to be related to the amounts of the drug given.

Individual susceptibility

Considering all dosages and patterns of injection employed, three monkeys exhibited affected cycles whenever they were treated during three or more cycles (monkeys IX, XIII, XVIII). Six monkeys, having received reserpine for two or more cycles, had 34 to 66% of the cycles prolonged, one had 80% and one had no cycles prolonged (even after treatment during 5 cycles). It may not be said with certainty from these few data that there is a varying degree of individual susceptibility to reserpine, although the fact that three monkeys were highly responsive, and four were substantially less sensitive, and one completely not, suggests that there are individual variations in responsiveness. If this is so, it is worth noting that the large dose given was always effective in the dose patterns during 1957–1958, except in monkeys V and XI.

Indices of ovarian function associated with reserpine injections

As noted under Methods, above, the Hartman vaginalse dimentation index (5) and differential staining of vaginal smears were employed continuously during the current study in order to assess ovarian function (see 6).

In monkeys which exhibited drug-associated uterine bleeding and lengthened menstrual cycles, atypical changes in the Hartman sedimentation index were often recorded. Figure 1 illustrates the difference in such indices between experimental and control menstrual cycles in two animals,

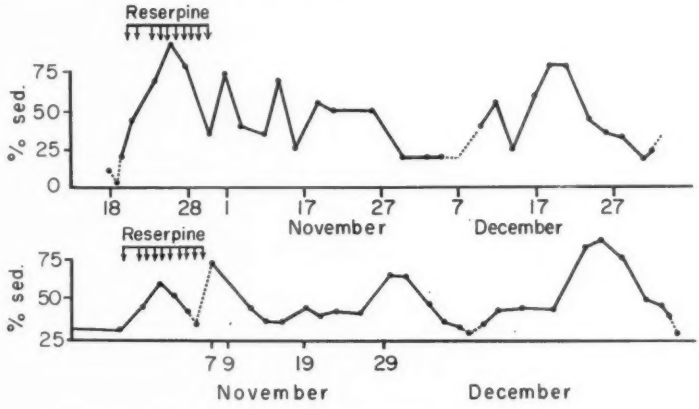

FIG. 1. Graph of heavy vaginal sloughing during and following a large dosage of reserpine (1 mg. per kg. per day) in one cycle (top) and light vaginal sloughing associated with a small dosage of reserpine (0.2 mg. per kg. per day) (bottom).

Note "breakthrough" uterine bleeding in bottom graph toward end of reserpine treatment.

one of which exhibited reserpine-associated uterine bleeding and lengthening of the cycle (XIX) and the other a lengthened menstrual cycle only (XI). These animals received reserpine, 1.0 mg. per kg. body weight, on Days 2 through 12.

Figure 2 illustrates the indices of ovarian function as revealed by the vaginal sedimentation and cytology in a representative monkey (VI) that are associated with reserpine injection, 1.0 mg. per kg. body weight daily from Days 7 to 12 of the menstrual cycle. The percentage of cornified cells in the vaginal smear as well as assessment of leucocyte-density based upon arbitrary grades (see 6) have been graphed, along with the Hartman sedimentation index.

It will be noted that a period of heavy vaginal desquamation occurred during, or just after, the period of drug administration. Periods of heavy vaginal desquamation occurred regularly in control cycles during the latter

half of the intermenstruum. Data which support the view that reserpine-associated periods of heavy vaginal exfoliation are related to estrogen-withdrawal are being published (L.B.E.). Drug-associated bleeding, when it occurs in the proliferative phase of the cycle is spoken of as "breakthrough" or interval-type bleeding, and is believed to be the result of estrogen-withdrawal.

The phenomenon of reduced sex skin swelling in animals which previously displayed it prominently was often noted during periods of heavy

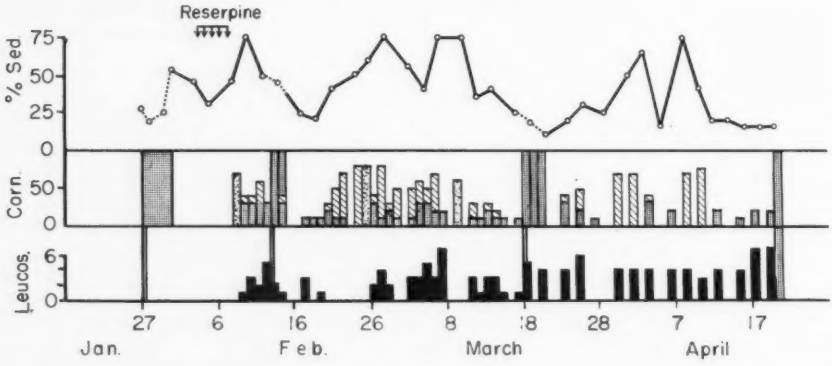

FIG. 2. Indices of ovarian function during reserpine treatment (1 mg. per kg. of body weight) and in a control menstrual cycle. The day of detection of onset of uterine bleeding is indicated by the vertical bars extending from the bottom to the second lines. The duration of uterine bleeding is shown by the broken line in the top graph.

Top, percentage of sedimentation in the vaginal lavage.

Middle, degree of vaginal cornification. Stippled areas, nucleated cornified cells. Mottled columns, clumping of epithelial cells. Striped columns, non-nucleated cornified cells.

Bottom, leucocyte count (black) in a paracervical vaginal smear, based on an arbitrary scale.

Note "breakthrough" bleeding several days after reserpine. Suppression of estrogen after the reserpine administration is suggested by this bleeding as well as by the presence of clumping and of nucleated, partially cornified cells, more than in the control cycle.

drug treatment. It may be mentioned that all six of the animals given the dosage regime indicated in Figure 1 showed evidence of an effect of reserpine both by lengthened menstrual cycles and drug associated "breakthrough" uterine bleeding.

Differences between the Hartman indices of experimental and control cycles in lightly reserpinized animals (dosage of 0.2 mg. per kg. body weight) from Days 2 through 12 of the menstrual cycle were not marked, and have not been illustrated. It may be mentioned in this respect that of the six monkeys in this experimental group, four showed no evidence of either drug-associated uterine bleeding or delay of menstruation.

DISCUSSION

The injection of reserpine in the several doses and patterns of administration used reveals several items of physiological import with respect to understanding the menstrual cycle in the monkey.

First, more than half of the cycles in treated monkeys were significantly prolonged when the 1 mg. per kg. of reserpine was given over three-day periods beginning on Days 3 to 9. Second, a lower dose (0.5 mg. per kg. of body weight) was more effective in this regard when given from Days 2 to 7 than the larger dose (1 mg. per kg. of body weight) given from Days 7 to 12. Finally, larger doses were far more effective in prolonging cycles than the smaller dose when the injection spanned days two to twelve.

It is clear that we did succeed in finding a very short, or critical time within the first part of the cycle when reserpine could so modify endocrine activity that the cycles would be prolonged. The dose had to be sufficiently large and span Days 2 through 7. This procedure may delay ovulation, although evidence of this has not been established in this work.

The matter of the extent of deviation of prolonged cycles from the means of the control cycles has been noted and found not to be related to the dose of reserpine. In only three monkeys were all cycles affected. It may be noted, with respect to monkey XIII that in the first cycle in which 0.2 mg. per kg. was given daily from Days 2 to 12, a delay in menstruation of 116 days was observed. This monkey seems, accordingly, to have been one which was distinctly susceptible to the action of reserpine, in contradistinction to the other monkeys of the series. Placebo injections of the reserpine vehicle alone given from Days 2 to 12 had no effect in this monkey in one cycle and in monkey X in two cycles (cycles 31 and 21 days in length, respectively).

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FURTHER OBSERVATIONS ON THE RELATIONSHIP BETWEEN SEROTONIN AND THE ADRENAL[1]

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ABSTRACT

Additional evidence for the profound influence of 5-hydroxytryptamine (serotonin) instituting a release of reducing material from the quartered rabbit adrenal has been accumulated. A high degree of specificity of chemical structure has been demonstrated by failure of the closely related 4- and 6-hydroxy isomers to mimic the effect of serotonin. In addition, removal or replacement of the 5-hydroxyl group with a fluorine atom destroyed activity. Marked decreases in activity were also obtained upon methylation of the amine group or substitution on the α-carbon. Concomitant introduction of amphenone and serotonin resulted in complete inhibition of the serotonin influence. On the other hand the activity of serotonin was protected by the monoamine oxidase inhibitor iproniazid. Evidence derived from the use of C^{14}-labeled serotonin, ultraviolet analysis and chromatography performed for detecting indole amines led to the conclusion that a metabolite of serotonin could not be responsible for the increased ability to reduce the blue tetrazolium reagent.

IT HAS been clearly demonstrated that serotonin has a direct influence on the quartered adrenals of several species tested (1). The level of reducing substances being elaborated by the gland markedly increased as visualized by the blue tetrazolium reaction. Although chemical characterization of the reducing material released by the serotonin stimulated adrenal has as yet proven elusive, additional information on the specificity of this adrenal response to serotonin has been obtained. In the previous paper (1) it was demonstrated that the rabbit adrenal was particularly responsive to 5-hydroxytryptamine (serotonin) and therefore adrenals from this species were exclusively used in the present study.

Experiments were designed to reveal structural specificity of the serotonin molecule and to permit observation of serotonin activity in the presence of certain enzyme inhibitors. Furthermore studies were devised to show as conclusively as possible that the reducing material elaborated from the adrenal was not a metabolite of the exogenous serotonin but an endogenous product of the gland.

It was found that the chemical structure of 5-hydroxytryptamine was extraordinarily precise for the requirement of gland stimulation. Displacement or substitution of the 5-hydroxyl group abolished practically all bio-

Received November 27, 1959.

[1] This work was supported by a grant number A-1207 from the Public Health Service

logical activity. Serious depression of the ability to form blue diformazan also resulted upon methylation of the amine group or alkylation on the α-carbon atom.

It was also observed that the monoamine oxidase inhibitor, iproniazid (2) augmented the effect of serotonin by virtue of preventing its destruction through monoamine oxidase action. On the contrary amphenone, an inhibitor of the hydroxylation reactions in the adrenal (3), was shown to eliminate the synthesis of reducing substances from the serotonin stimulated gland. Finally the results of a number of experiments conclusively disproved that a metabolite of the exogenous serotonin was responsible for the total reducing content of the medium bathing the adrenals.

METHODS AND EXPERIMENTAL

The procedures utilized in this work were those reported in a previous paper (1). The compounds tested for their capability to release blue tetrazolium reactive compounds from the adrenal were studied on an equimolar basis with serotonin in Krebs-Ringer phosphate buffer. For these comparative studies 4 compounds and serotonin were tested simultaneously on the adrenals from 25 rabbits. The glands were quartered and divided among five flasks in such a manner to reduce biological variation. Each flask contained 3 ml. of phosphate buffer per pair of adrenals and the test substance was added in a 0.1 ml. volume. The first hour of incubation was used to obtain control levels and the test compounds were compared to serotonin over the next 1½ hours.

In the experiments with amphenone hydrochloride [1,2-bis (p-aminophenyl)-2-methylpropanone-1 dihydrochloride] the tissue was also divided and incubated as above so as to maintain a representation of each animal in each flask. This permitted more accurate determination of the influence of graded doses of amphenone and the ability of serotonin to overcome the inhibition induced by amphenone. The contents of each flask were analyzed for reducing material after a 1 hr. control incubation period, after a 1½ hr. exposure to serotonin plus amphenone in fresh medium and after 1½ hrs. in fresh medium with additional serotonin.

Both amphenone and iproniazid (marsilid, 1-isonicotinyl-2-isopropyl hydrazine) were tested for reactivity with the blue tetrazolium reagent and recovery studies were conducted to correct for any interference by these substrates. A similar study was performed on 4- and 6-hydroxytryptamine.

In all experiments the incubation medium was extracted 3 times with 30 ml. methylene chloride, the solvent removed under vacuum and the residue dissolved in ethanol. Suitable aliquots were employed in the determination of the quantity of blue diformazan produced. The blue tetrazolium procedure has been shown to have a sensitivity of one μg. and a reproducibility for tissue of 2 to 3 μg.

In several instances serotonin metabolites were searched for by chromatographing the residues from the methylene chloride extracts in the two-dimensional system of Jepson (4).[2] To some of the residues a known quantity (usually 100 μg.) of cortisone and hydrocortisone were added in order to determine the specificity of the Jepson system and the Ehrlich reagent for indole amines.

C^{14}-serotonin labelled in the β-carbon was also used to gather additional information as to the nature of the material capable of reducing blue tetrazolium found in the incu-

[2] We wish to express our thanks to Dr. A. Feldstein for putting his chromatographic equipment and methods at our disposal and for samples of iproniazid, isocarboxazid (marplan) and isonicotinyl hydrazine.

bation medium. A Tracerlab automatic flow counter was employed to measure the total counts recovered in the methylene chloride extracts as well as the counts in the eluates obtained after silica gel chromatography (1).

Finally efforts were made to exhaust the adrenal glands of their normal complement of reducing material by incubating the tissue for 3 hrs. without serotonin. The medium was changed at 1 hr. intervals and the serotonin was added for a 4th hr. incubation. The residues of the methylene chloride extracts were examined in ethanol in a Cary Model 11 ultraviolet spectrophotometer.

RESULTS

The high degree of specific structural requirements to evoke an *in vitro* release of reducing material from the rabbit adrenal can be seen in the data of Table 1. Substitution of the 5-hydroxyl group (compound 3) or its displacement to an adjacent position (compounds 4 and 5) practically eliminated all activity of the molecule. Methylation of the amine group (compound 2) diminished activity by approximately 50%. A more accurate estimation of the influence of bufotenine was difficult because of its limited solubility. However serotonin was prepared in a similar suspension to that used for bufotenine and here again the dimethylated analogue was about half as active as serotonin.

The necessity of an accessible amine group is probably also revealed by the finding on α-methylserotonin (compound 6). Addition of a methyl group to the carbon adjacent to the amine unit severely diminished the activity of the serotonin molecule.

The apparent slight activities of compounds 7 and 8 probably arose from

TABLE 1. THE SPECIFICITY OF THE SEROTONIN REACTION ON RABBIT ADRENAL

Compound*	Blue diformazan formed above control level†	Relative activity
	μg./10 adrenals	
1. 5-Hydroxytryptamine	286	100
2. Bufotenine (N,N-dimethylserotonin)[a]	143‡	50
3. 5-Fluorotryptamine[b]	6	2
4. 6-Hydroxytryptamine[c]	9	3
5. 4-Hydroxytryptamine[c]	6	2
6. α-Methylserotonin[a]	3	1
7. α-Methyl-5-hydroxytryptophane[a]	14‡	5
8. 5-Hydroxyindoleacetic acid	11‡	4
9. N,N-dimethyltryptamine[a]	3	1
10. 3-(3-dimethylamino-2-methyl)-propylindole[a]	6‡	2
11. γ-Dimethyltryptamine[a]	3‡	1
12. 1,α-Dimethyltryptamine[a]	9	3
13. Tryptamine acetate[a]	27	9
14. α-Ethyltryptamine acetate[a]	+	+
15. α-Methyltryptamine acetate[a]	+	+

* We wish to thank the following for samples of the compounds tested: [a] Dr. R. V. Heinzelman of the Upjohn Co. for compounds 2, 6, 7, 9–15; [b] Dr. Ernst D. Bergmann for compound 3; and [c] Dr. Albert Hofmann of Sandoz Co. for compounds 4 and 5. Equimolar concentrations of the compounds were tested.

† Control levels range between 12 to 20 μg. per 10 adrenals.

‡ These values may actually be higher since it was necessary to add these compounds in suspension.

+ Inhibition occurred.

TABLE 2. RECOVERY STUDIES AND REACTIVITY OF SEROTONIN ISOMERS WITH BLUE TETRAZOLIUM

Compound*	Quantity added	Quantity recovered	Optical density of the blue diformazan formed by 10 μg. of compound
	μg.	μg.	
4-Hydroxytryptamine	1440	200	0.74
5-Hydroxytryptamine	1390	10	0.64
6-Hydroxytryptamine	930	7	0.95

* These solutions were assayed immediately after preparation.

small quantities of these substances being extracted into the methylene chloride phase. The hydroxy indole structure is known to react with blue tetrazolium (1, 5). The small degree of activity discerned for compound 13 was unexpected since no blue tetrazolium reactive group is present. Since compound 13 led to a yellow colored methylene chloride extract, it was reasonable to assume the presence of a methylene chloride-soluble impurity that may have reacted with the blue tetrazolium reagent.

Further examination of the hydroxytryptamine isomers was carried out in order to establish the extent of interference by these compounds because of their ability to produce formazans. Their extractibility into methylene chloride and their reactivity with the blue tetrazolium reagent are listed in Table 2. Only 4-hydroxytryptamine has a significant solubility in methylene chloride and a correction of 14% has been applied in studies using this isomer.[3]

It is of interest to mention here that equivalent quantities of the hydroxytryptamine isomers in freshly prepared solutions gave different optical densities with the blue tetrazolium reagent (Table 2, last column) which may be useful in characterizing them. The protection of solid samples of the 5- and 6-isomers afforded through the creatinine sulfate complex and the 4-isomer through oxalate formation was lost in 80–90% alcoholic solution. Deterioration (as measured by diformazan formation) of hydroxytryptamine solutions commenced in the first day and continued for several days. Fifty per cent reactivity with the blue tetrazolium reagent was lost in 10 days in the case of serotonin.

The recovery experiments with serotonin provided the first suggestions that a methylene chloride-soluble metabolite of 5-hydroxytryptamine was not responsible for the increased formation of blue diformazan (1). Experiments were devised to obtain unequivocal proof for this supposition. One such study employing C^{14}-labelled serotonin led to the results given in Table 3. Less than 5% of the added radioactivity was recovered in the organic solvent. Moreover, on the average less than 1% of the added counts appeared in the peak eluate after silica gel chromatography.

[3] 4-Hydroxytryptamine caused a rich blue staining of the adrenal tissue. The dye could not be extracted with most of the usual organic solvents.

It one calculates the actual contribution of the blue diformazan produced by the serotonin recovered in the methylene chloride extract after incubation with tissue, one finds that 80% of the reducing material must have been of tissue origin. For example it can be seen from the data in Table 3 that on the average 2.8% of the added serotonin (total counts recovered divided by total counts added) would augment the blue diformazan levels. Where 6.87 mg. of serotonin was used in experiment one, 110 μg. of serotonin (6.87 mg. multiplied by 1.6% recovery value) would appear in the final measurement of reducing material in the extract. A similar calculation for the other 3 experiments reveals that the recoverable serotonin can only account for approximately 20% of the total blue diformazan formed.

A second study to clarify the issue concerning a metabolite of 5-hydroxy-

TABLE 3. RECOVERY OF METABOLITES OF RADIOACTIVE SEROTONIN

Exp. No.	Weight added*	Total counts added	Total counts recovered	Recovery	Blue diformazan arising from serotonin recovered	Corrected blue† diformazan formed after incubation
	mg.	*G*		%	μg.	μg.
1	6.87	642,000	10,000	1.6	110	579
2	9.16	856,000	38,000	4.4	394	2014
3	10.99	1,027,000	26,000	2.5	274	825
4	13.73	1,284,000	33,000	2.6	357	1100

* Weight refers to 5-hydroxytryptamine creatinine sulfate.

† The number of rabbit adrenals employed were 40 for experiment 1, 60 for 2, 50 for 3 and 60 for 4.

tryptamine was explored through the introduction of iproniazid. It was reasoned that inhibition of the adrenal monoamine oxidase would prevent formation of serotonin derivatives. Preservation of the serotonin moiety might also be reflected in a prolonged or increased stimulation of the adrenal. The results of this study are enumerated in Table 4. Unfortunately it was discovered that iproniazid itself was partially soluble in methylene chloride and was very reactive with the blue tetrazolium reagent. In order to apply sensible corrections for the interference of iproniazid in formazan production, recovery experiments of iproniazid phosphate were carried out side by side with monoamine oxidase inhibition.[4]

The first and last figures in column 3 of Table 4 demonstrate that the recovery of iproniazid phosphate amounted to 14.2% irrespective of the presence or absence of tissue. Therefore the 284 μg. due to iproniazid phos-

[4] Because of the importance of the blue tetrazolium reaction in steroid analysis, the curiosity stimulated by the reactivity of iproniazid prompted a short excursion in search of the structural entity responsible for the positive reaction. It was found that the hydrazine unit alone would give rise to a formazan. The optical densities obtained with 10 μg. of compound were as follows: iproniazid, 0.55; isonicotinylhydrazine, 0.60; isocarboxazid (marplan) [1-benzyl-2-(5-methyl-3-isoxazolylcarbonyl) hydrazine], 0.65; semicarbazide, 0.61; phenylhydrazine, 2.05; nicotinamide and nicotinic acid, no reaction.

phate were subtracted from the total of the diformazan formed of column 3 yielding the corrected levels of blue diformazan in column 4. It can now be seen in the last column that iproniazid phosphate protected serotonin. At each of the concentrations of serotonin a greater effect was observed in the presence of iproniazid phosphate. As pointed out under methods the blue tetrazolium procedure was reliable to a change of 2 μg. Lines 1 and 2 of the table reveal that iproniazid phosphate itself has no influence on the release of reducing material from the adrenal.

Another piece of evidence eliminating a serotonin metabolite as the cause of the increased titers of material capable of producing blue diformazan was gained from chromatography studies. The residues from sufficient aliquots of the methylene chloride extract were chromatographed in

TABLE 4. THE INFLUENCE OF IPRONIAZID PHOSPHATE ON FORMATION OF MATERIAL YIELDING BLUE DIFORMAZAN

Marsilid added	Correction for iproniazid interference	Total blue diformazan formed	Corrected levels of blue diformazan	Added serotonin	Increase due to serotonin
mg.	%	μg./10 adrenals	μg./10 adrenals	μg./10 adrenals	μg./10 adrenals
2.0	14.2	300*		0	
0		16	16	0	
0		23	23	20	7
2.0		315	31	20	15
0		36	36	200	20
2.0		420	136	200	120
0		43	43	1000	27
2.0		630	346	1000	330
2.0‡	14.2	285†		0	

* Control levels of material that reduce blue tetrazolium were between 15–18 μg./10 adrenals.

† Used to calculate recovery of iproniazid phosphate and derive per cent correction (column 2).

‡ No tissue present.

the indole amine system of Jepson (4). In no instance was a positive reaction elicited for indole amines by the Ehrlich reagent. In a separate experiment 100 μg. amounts of cortisone and hydrocortisone were deliberately added to the residues from the methylene chloride extracts and also chromatographed in the indole amine system. No blue tetrazolium reactive zones could be detected on the paper suggesting that the steroids had run off or decomposed.

In an attempt to study the interval of responsiveness of the adrenal to serotonin a sequence of control incubations were performed and culminated with a delayed exposure to 5-hydroxytryptamine. The results in Table 5 are an outline of 4 such experiments. In every case the adrenal gland responded to serotonin after 3 hrs. of incubation without serotonin. This afforded a procedure for removal of the majority of the endogenous blue tetrazolium reactive material being secreted by the glands. At the same

TABLE 5. SEROTONIN EFFECT ON RABBIT ADRENAL AFTER EXHAUSTION OF REDUCING MATERIAL IN THE GLAND

No. of glands	Incubation period	Addition of serotonin	Total blue diformazan formed
	hr.	mg./pr. adrenals	μg.
62	1st	0	216
62	2nd	0	70
62	3rd	0	41
62	4th	0.5	2815
56	1st	0	223
56	2nd	0	62
56	3rd	0	56
56	4th	0.5	2013
54*	1st	0	200
54*	2nd	0	100
54*	3rd	0	50
54*	4th	0.5	730
52*	1st	0	131
52*	2nd	0	80
52*	3rd	0	58
52*	4th	0.5	550

* These animals had been previously treated with a variety of steroids in another laboratory. The blue diformazan levels have not been corrected for serotonin interference.

time dependable measurements on the residues could be carried out in the ultraviolet region.

5-Hydroxytryptamine creatinine sulfate, used as a reference substance, was found to have 2 absorption bands in 90% ethanol. A band near 275 mμ with an $\epsilon = 5{,}480$ (for the complex) was the expected absorption of the indole amine. The second band near 225 mμ had an $\epsilon = 25{,}400$ and originated from the creatinine moiety. Creatinine itself gave this band near 235 mμ with an $\epsilon = 8{,}430$, while creatinine sulfate and creatinine hydrochloride demonstrated that a change of pH toward the acid side resulted in the shift of the 235 mμ band to shorter wavelengths (e.g. 225 mμ). Calculations of the ϵ for the 225 mμ band in the spectrum of 5-hydroxytryptamine creatinine sulfate just on the basis of the molecular weight of creatinine yields a value near 8400.

Examination of the residues from incubations without serotonin yielded the expected absorption near 240 mμ. for steroids containing a conjugated 3-ketone. The ultraviolet spectrum obtained on the residue from a serotonin incubation yielded bands near 275 and 225 mμ. Not all of the 275 mμ absorption could be associated with that at 225. Employing these bands for calculation of the quantity of serotonin recovered from the incubation, values of 2% were obtained. When similar calculations to those on p. 836 are carried out, it is again found that 2% of the added serotonin only augments the blue diformazan titer obtained after incubation with tissue by 20%. The remaining 80% must be of adrenal origin. It was of interest to note that the serotonin was recovered as the creatinine complex since the short wavelength absorption occurred and was near 225 mμ.

In another direction of research the inhibitor of hydroxylation reactions, amphenone, was examined for its effect on serotonin activity. The results of this exploration are given in Table 6. It can be seen from this data that 50 μg. of amphenone were sufficient to bring forth a 50% inhibition of the serotonin stimulation. Little reversibility of the inhibition induced by amphenone could be achieved by a second incubation with additional 5-hydroxytryptamine unless the ratio of serotonin to amphenone exceeded 10.

In another experiment it was confirmed that no release of reducing substances was brought forth by serotonin from liver or kidney. This has now also been found for heart and skeletal muscle examined under identical conditions to those employed for the adrenal glands.

DISCUSSION

The high degree of structural specificity observed for the interaction of 5-hydroxytryptamine with the rabbit adrenal encouraged the conclusion of a fundamental role for serotonin in this gland. That amphenone interrupted the mechanism through which serotonin operated suggested that the influence of 5-hydroxytryptamine was close to or dependent upon biogenetic steps involving hydroxylation. A similar thesis was established by Rosenfeld and Bascomb (3) for the interrelationship of amphenone and adrenocorticotropic hormone in the perfused adrenal.

From the results of this present study it appeared certain that the reducing material elaborated by the adrenal upon stimulation by serotonin could

TABLE 6. THE INHIBITION OF SEROTONIN STIMULATION OF THE ADRENAL WITH AMPHENONE

Content amphenone	1st incubation			2nd incubation		
	Serotonin content	Blue* diformazan formed	Inhibition	Serotonin added	Blue diformazan formed	Response recovery
μg.	mg.	μg./10 adrenals	%	mg.	μg./10 adrenals	%
0	0.5	200		0.5	68	
10	0.0	120	40	0.5	67	100
10	0.5	130	34	0.5	69	100
800+1 mg. ACTH	0.5	50	75			
0	0.5	220		0.5	74	
0.1	0.5	210	6	0.5	74	100
1.0	0.5	190	12	0.5	73	100
10.0	0.5	160	25	0.5	75	100
50	0.5	110	50	0.5	25	34
132	0.5	31	86			
0	0.5	288		1.0	93	
500	0.5	13	91	1.0	13	14
1000	0.5	10	92	1.0	16	17
2000	0.5	12	93	2.0	15	16
3000	0.5	11	93	3.0	13	14

* Control levels were 12 μg./10 adrenals and the blue diformazan values have been corrected for serotonin recovery.

not be a metabolite of 5-hydroxytryptamine. Moreover hydroxylation of the indole nucleus would have depreciated the molecule's solubility in methylene chloride. Furthermore chromatographic fractionation failed to uncover substances with an intact indole nucleus. Introduction of an active reducing group on the side chain could be eliminated since the extent of recovery of C^{14}-labeled serotonin could not account for more than 20% of the total blue diformazan formed. These overall findings were clearly substantiated by the ultraviolet analyses. Calculations based on the creatinine and indole absorption bands established that only 20% of the total blue diformazan produced could arise from the added serotonin.

Although the results with iproniazid phosphate were not unexpected, it was of interest to note that the adrenal monoamine oxidase could be inhibited. The inactivation of this enzyme system clearly afforded maintenance of serotonin concentrations.

The experiments on "exhausted" adrenal glands yielded two helpful facts. Firstly that the normal or usual complement of reducing material readily secreted by the gland could be successfully removed without disturbance of the response to serotonin. This will now permit the stockpiling of the labile compounds elicited by serotonin in a less contaminated form. Secondly, it was possible to distinguish between previously steroid-treated and untreated animals. Usually the rabbits that were treated with steroids had received either progesterone or synthetic steroids being screened for progestational activity. Whereas progesterone was used as a subcutaneous standard, 19-nor-ethynyl testosterone was used as an oral standard. In most instances, the adrenals appeared smaller for all groups of steroid-treated animals as compared to the adrenals from the non-treated groups. Recently Edgren *et al.* (6) have demonstrated that progestational hormones cause adrenal atrophy. In every case of steroid-treated rabbits diformazan production was significantly reduced in each incubation period without serotonin. Moreover the response to serotonin was noticeably diminished.

Addendum

At the completion of this manuscript a sample of a human adrenal tumor became available. The tissue appeared cortical in nature and 1.5 gm. of slices were incubated under the same conditions like those used for the rabbit adrenals. At the termination of a 1 hr. control incubation period, the medium was extracted as described previously and 28 μg. of blue diformazan was formed. The tissue was re-suspended in fresh medium containing 15 mg. serotonin creatinine sulfate and re-incubated for an additional hour. At the end of this interval the medium yielded 900 μg. of reducing substances. Examination of the ultraviolet spectrum revealed the presence of the 225 and 275 mμ absorption bands of serotonin creatinine sulfate. Using the densities of these bands it was possible to estimate that only 20% of the

total diformazan producing material from the serotonin incubation could have originated from the added serotonin.

Acknowledgment

We wish to express our sincerest gratitude to Dr. Marcus M. Mason for having his group stockpile rabbit adrenals for us. Grateful appreciation also goes to Susan Mandell, Thomas A. Barry and Matthew Connors for technical assistance.

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GONADOTROPIN AND ADRENOCORTICOTROPIN FROM RAT PITUITARY HOMOGRAFTS AS MANIFESTED BY HOST RESPONSE TO CHORIONIC GONADOTROPIN AND AMPHENONE

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ABSTRACT

Hypophysectomized rats bearing 4 anterior pituitaries grafted under the renal capsule exhibit an adrenomegalic response to amphenone, an effect which is lacking in hypophysectomized controls. Such graft-bearing rats also show an ovarian response to administered human chorionic gonadotropin markedly in excess of the maximum response obtainable in hypophysectomized controls. These data indicate substantial adrenocorticotropin and gonadotropin production and release from rat pituitary tissue having no physical hypothalamic connections. Moreover, since the adrenomegalic response to amphenone is mediated through suppression of corticoid production by the rat adrenal, these pituitary grafts apparently also maintain a degree of reciprocal functional relationship with the adrenal cortex.

IN AN accompanying paper we have presented evidence for the continued secretion and release of growth hormone by pituitary grafts in the hypophysectomized rat (1). This report describes experiments in which the respective ovarian and adrenal responses to administered chorionic gonadotropin and amphenone in hypophysectomized rats bearing pituitary grafts indicate that such grafts are capable of producing and releasing both gonadotropin and adrenocorticotropin.

MATERIALS AND METHODS

Weanling Sprague-Dawley rats, 28–30 days of age, were hypophysectomized by the parapharyngeal approach and 2 days later half of them received 4 whole pituitary grafts under the left kidney capsule from donors of the same sex and age. Maintenance and feeding conditions have been previously described (1). From 3 weeks to 3 months later the respective adrenal and ovarian responses of the grafted and hypophysectomized controls were tested by the administration of either 0.2 mgm. per kg. oral amphenone in 0.25 cc H_2O daily for 14 days or by administration of 20 I.U. human chorionic gonadotropin subcutaneously daily for 5 days in 0.2 cc H_2O. The rats were sacrificed 24 hours following the last of these treatments and the quantitative data recorded in Table 1 were obtained. The sella of each animal was carefully examined under good illumination and ten-fold magnification for completeness of the hypophysectomy and the grafts were recovered for histological study.

Received November 30, 1959.

RESULTS AND DISCUSSION

Prior studies have established that the adrenomegalic effect of amphenone results from apparent increase in ACTH release from the anterior pituitary resulting from lowered output of corticoid by the adrenal. Hence, in the hypophysectomized rat such adrenomegalic effect is totally lacking (2). The present data confirm these observations in the hypophysectomized rat. However, substantial adrenomegalic effect accompanied by the highly characteristic yellowish discoloration of the adrenals is observed in the hypophysectomized-grafted rat. This effect demonstrates that the ectopic pituitary without any hypothalamic connection can respond at least to a limited degree to an amphenone-induced lowering of adrenal hormone output by increased ACTH release.

The precautions we have taken to overcome quantitative limitations in the amount of available ectopic pituitary tissue by utilizing 4 pituitary glands may account for the positive effects we have obtained. Also stressful stimuli utilized by others represent a more acute demand for ACTH release than does the chronic administration of amphenone. Nevertheless, it is clear that a quantitatively limited but qualitatively distinct reciprocal working relationship between adrenal and pituitary can be demonstrated under our experimental conditions.

Reichert *et al.* (3) showed that the ovarian response of the immature hypophysectomized rat to chorionic gonadotropin is quantitatively very limited. Thus, ovaries weighing more than 30 mg. can not be produced in the completely hypophysectomized immature rat. Our data substantiate this. Moreover, it is clear that the ectopic pituitary tissue can release sufficient pituitary gonadotropin to markedly augment the biological response to the administered chorionic gonadotropin (4). This is also apparent from the greater weights of ovaries and uterus in the hypophysectomized-grafted rat compared with those of the hypophysectomized animal. Hence we may conclude that in the absence of any hypothalamic connection rat pituitary tissue is capable of producing and releasing readily detectable amounts of biologically active gonadotropin.

The data in Table 1 reveal also that the expected goitrogenic effect of amphenone is totally lacking in the hypophysectomized as well as in the hypophysectomized-grafted animal. Histological examination of the thyroid glands of these animals also fails to reveal clear evidence of thyrotropin response. However, Scow and Greer have shown that the thyroid-serum iodide ratio is above that of the hypophysectomized mouse in graft-bearing hypophysectomized mice treated with propylthiouracil (5). Hence the thyrotropic potential of ectopic pituitary tissue merits further study.

The data presented in a preceding paper (1) and in this report clearly indicate that the anterior pituitary continues to function to a substantial degree after complete separation from physical connection with the hypo-

TABLE 1. RESPONSE TO AMPHENONE OR CHORIONIC GONADOTROPIN IN HYPOPHYSECTOMIZED-GRAFTED VS. HYPOPHYSECTOMIZED ANIMALS*

Endocrine status	Body wt. (gms.)	Ovaries (mgm.)	Adrenals (mgm.)	Thyroid (mgm.)
Hypox	90 ± 4	4 ± 0.5	10 ± 1	4 ± 0.5
Hypox + grafts	138 ± 5	9 ± 1.5	12 ± 2	6 ± 1
Intact	184 ± 12	67 ± 9	53 ± 6	11 ± 2
Hypox + amphenone	90 ± 7	4 ± 0.7	10 ± 0.8	4 ± 0.6
Hypox + grafts + amphenone	128 ± 11	10 ± 1.0	28 ± 3	5 ± 0.8
Intact + amphenone	177 ± 11	70 ± 11	71 ± 5	34 ± 5
Hypox + HCG†	92 ± 5	23 ± 4	—	—
Hypox + grafts + HCG	130 ± 9	143 ± 15	—	—

* Data from a representative series presented; 61 days of age at autopsy; each series repeated 3 times with equal numbers of rats; 8 animals per group; high mortality in amphenone-treated hypophysectomized rats.

† HCG = Human chorionic gonadotropin.

thalamus. However, it is also clear that the quantitative level of somatotropin, adrenotropin and gonadotropin produced and released under these conditions is not adequate for optimal endocrine function. Hence, the deficiency appears to be relative rather than absolute and one is led to attribute a substantial degree of hypothalamic independence to the anterior pituitary. In fact, a small number of grafted-hypophysectomized male rats retain active spermatogenesis (1, 6). Since a comparable phenomenon has not been seen in the female, it may be inferred that more complete hypothalamic dependency is involved in meeting the greater quantitative demands of critical phases of the ovulatory cycle. In any event, it is clear that a basal level of anterior pituitary function continues without physical connection with the hypothalamus.

Acknowledgment

The highly skilled technical assistance of Mr. Howard Erwin and Mr. Charles Turner is gratefully acknowledged.

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CHEMICAL IDENTIFICATION OF IODINE-CONTAINING COMPOUNDS IN HUMAN SERUM[1]

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ABSTRACT

Studies have been made on the extraction of iodinated compounds from human serum, their chromatographic separation on filter paper, and their detection by a ceric sulfate-arsenious acid reagent, followed by staining with aniline in acetone and neutralizing with sodium bicarbonate. Chloride simulated iodine compounds on chromatograms. Thiouracil, thiouracil disulfide and propylthiouracil were found to reduce ceric ion catalytically and when run in certain solvent systems moved to positions which could cause confusion with the iodotyrosines or iodothyronines. Only thyroxine and iodide were regularly identified in normal human serum, but small amounts of unknown positive-reacting compounds were found among patients with simple goiter.

WHEN Bowden, Maclagan and Wilkinson (1) adapted the catalytic ceric-arsenious reaction of Sandell and Koltoff (2) to the detection of minute amounts of iodine-containing compounds on filter paper, a sensitive method became available which has been widely applied to the detection and measurement of iodine compounds in tissues and body fluids following chromatography or electrophoresis (3–6). Minor modifications of the original technique have been introduced to intensify the spots, to increase sensitivity and to preserve the chromatogram (7–9); but no systematic study seems to have been made of substances which might interfere with the test by simulating iodine in the reaction.

Identification of the iodine-containing compounds in human serum without the use of radioiodine would facilitate studies of thyroid function and aid in the diagnosis of patients suffering from thyroid disease. Several procedures have been devised for concentrating the compounds which might

Received January 8, 1960.

[1] This study was supported by grants from the National Institutes of Health, United States Public Health Service (No. A-612), and from the American Cyanamid Company.

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reasonably be expected to be found in serum and freeing them sufficiently from salts and extraneous substances to permit chromatographic analysis on paper (3, 4, 10–12). The studies to be described were undertaken in an attempt to devise a workable procedure and to determine the factors which might lead to misleading conclusions.

EXPERIMENTS AND RESULTS

Detection of iodinated compounds on paper chromatograms. Various amounts of iodinated compounds between 1.0 and 0.001 μg. were spotted on Whatman No. 3 MM paper in an area of about 0.25 cm^2. After drying, the papers were stained with the mixture of equal volumes of 10% ceric sulfate and 5% arsenious acid in 1N sulfuric acid solution for 30 minutes at room temperature as described by Bowden, Maclagan and Wilkinson (1). Iodinated compounds gave rise to white spots on a yellow background. After being dried the papers were dipped into a 3% solution of aniline in acetone (9) and dried again, whereupon the color of the background changed to green. The papers were then dipped into a saturated aqueous solution of sodium bicarbonate to neutralize the acid and then dried again. A permanent record was thus obtained; the color of the background became blue and contrasted sharply with the white spots. The minimal detectable amounts of iodinated compounds on 0.25 cm^2. of paper by this procedure were as follows: thyroxine (T_4) 0.005 μg., 3,5,3′-triiodothyronine (T_3) 0.008 μg., 3,5-diiodothyronine (T_2) 0.01 μg., 3,5-diiodotyrosine (DIT) 0.001 μg., 3-monoiodotyrosine (MIT) 0.002 μg., potassium iodide (KI) 0.001 μg. Because of diffusion during development larger quantities were needed for chromatography but quantities of 0.1 μg. of T_4, 0.1 μg. of T_3, 0.02 μg. of DIT, 0.02 μg of MIT and 0.01 μg. of potassium iodide were clearly detected on chromatograms after development in the following solvent systems:

	Ref.
BAW system—N-butanol-acetic acid-water (75:10:15)	(13)
BEAa system—N-butanol-ethanol-2N-ammonium hydroxide (5:2:1)	(11)
BEAb system—N-butanol-ethanol-2N-ammonium hydroxide (5:1:2)	(14)
2BA system—2-butanol-3% ammonium hydroxide (3:1)	(5)

Separation of iodinated compounds from human serum. The method used was adapted from several previously published procedures (15, 16, 3–5, 9–12), and in its final form most closely followed that of Block, Werner and Mandl (10, 5). Five to 10 ml. of human serum was extracted with 2.5 volumes of N-butanol saturated with 0.1N sulfuric acid and then twice with N-butanol by shaking vigorously for 5 minutes and centrifuging. The combined acid butanol solution was made alkaline to bromthymol blue with 2N ammonium hydroxide and evaporated in vacuum in a flash evaporator at less than 40° C. to a volume of about 1 ml. This dry butanol solution was filtered through a 1.2 cm. diameter filter paper on a microfilter using slight suction, and the flask was washed with 3 ml., and then with 1

ml. of dry butanol. To the combined filtrates an equal volume of chloroform was added, and this mixture extracted once with an equal volume and twice with one-half volumes of 2N ammonium hydroxide; gentle mixing avoided troublesome emulsions difficult to break by centrifugation. The ammonia solutions were evaporated in vacuum and the residue transferred to paper using 0.3 ml. and 0.2 ml. portions of methanol containing 1% concentrated ammonia. Usually half of the final residue was applied as a straight line 1.0 to 2.5 cm. long to a strip of Whatman No. 3 MM paper and developed in the BAW system and another half in one of the BEA systems. After 16 hours' development with ascending technique at room tempera-

TABLE 1. R_f'S OF COMPOUNDS WHICH REACT WITH THE CERIC-ARSENITE REAGENT IN FOUR CHROMATOGRAPHIC SOLVENT SYSTEMS

Solvent	T_4	T_3	T_2	DIT	MIT	KI	NH_4Cl	NaCl	TU*	TU-DS†	PTU‡
BAW	0.84 to 0.78	0.84 to 0.78	0.84 to 0.78	0.56	0.45	0.18 to 0.13	0.10 to 0.086	0.07 to 0.058	0.57 and 0.54 and 0.50	0.57	0.80
BEAa	0.27 to 0.20	0.50 to 0.43	0.51 to 0.45	0.05 to 0.037	0.10 to 0.085	0.34	0.16	0.12			
BEAb	0.53 to 0.52	0.66		0.26 to 0.15	0.26 to 0.17	0.44	0.20 to 0.14	0.18 to 0.14	0.37 and 0.34 and 0.30	0.29	0.64
2BA	0.40	0.53	0.56	0.07 to 0.064	0.10	0.32	0.17 to 0.14	0.14 to 0.11	0.22 and 0.13 and 0.069	0.16 and 0.064	0.53

* Thiouracil.
† Thiouracil-disulfide.
‡ Propylthiouracil.

ture the chromatograms, about 30 cm. long, were dried in a current of air and stained as already described.

The iodinated compounds were identified from their R_f's in both the BAW system and one of the BEA systems using the values shown in Table 1.

Interference by salts. The various steps in the extraction procedure were arrived at largely because alternative methods failed to reduce the salt content to a point where the running of the chromatogram was not disturbed. Direct extraction of the butanol extract of serum with ammonia left sufficient salt to cause two liquid phases to form on the chromatogram as well as spurious white spots and distortion of the R_f's of known compounds. Addition of chloroform to the butanol facilitated extraction and obviated lipid material which otherwise appeared near the front of the chromatogram. It was further determined that it was essential that salts be further excluded by evaporating the butanol to a small volume to remove water and filtering.

Substances giving spurious reactions for iodine. As noted above, chloride ion yielded a white spot upon staining, as little as 5 μg. being detectable. In general chloride caused bleaching of the ceric sulfate at a much slower rate than iodine-containing compounds and 5 μg. took as long as 15 minutes be-

fore being detectable. Larger amounts caused more rapid fading and 50 μg. was detected in 3 minutes. While iodine in usual quantities bleached in seconds, the reaction had to be allowed to proceed for 30 minutes when minute amounts were to be detected.

Besides giving rise to confusion, unless recognized, chloride had the additional unfavorable property of causing the bleaching to spread to adjacent areas of the chromatogram, thus obscuring other spots. Moreover, when sodium and ammonium chlorides were chromatographed in certain solvent systems, the R_f's were such as to mimic certain iodine-containing compounds (Table 1). In the BAW systems chloride ran with an R_f of 0.1 or less and this area of the paper could be cut off before staining.

Thiouracil, propylthiouracil and related substances have often been used as reducing agents and added to serum before processing to avoid exchange of radioiodine or deiodination. It was noted that when spotted on paper these compounds yielded white spots upon staining, as little as 4 μg. being easily detectable. That this was not simply a direct reduction of the ceric sulfate was shown when upon omitting the arsenite from the reagent no spots appeared until large quantities of the compounds were used.

Still more disturbing was the finding that the R_f's of these compounds were such as to give rise to confusion with the known iodine-containing compounds. Solutions of thiouracil and propylthiouracil when added to butanol and carried through the extraction procedure yielded one or more spots, depending on the solvent system, with the R_f's shown in Table 1. Accordingly, the disulfide of thiouracil was prepared in the usual way (17) but using sodium hypochlorite instead of iodine as oxidant. The R_f's of the disulfide corresponded to one of the spots which appeared in chromatograms of aged solutions. A solution of as little as 1 mg. of thiouracil carried through the extraction procedure gave rise to the two spots referred to.

In the BAW system thiouracil and its disulfide were similar in R_f to diiodotyrosine while the R_f of the propyl derivative was similar to that of T_4, T_3 and T_2. In the BEAb system thiouracil appeared between iodide and MIT or DIT and the disulfide a little above the iodotyrosines; propylthiouracil migrated to a position near T_3. In the 2BA system thiouracil could be confused with DIT or MIT, while the disulfide was indistinguishable from DIT; propylthiouracil could be confused with T_3. A great many organic compounds when used in large amounts gave rise to white spots by directly reducing ceric ion. Of the 20 amino acids tested in amounts of 10 μg., only methionine, cystine and tyrosine gave white spots while tryptophane stained a reddish color (Table 2).

Of the 21 sera from 18 individuals thus far processed by the method described, substances corresponding in R_f to thyroxine and iodide in two or more solvent systems have uniformly been detected. The iodotyrosines were not identified with certainty in any instance. Tryptophane was some-

TABLE 2. R_f VALUES OF FOUR POSITIVE-REACTING AMINO ACIDS

Solvent	*l*-methionine	*l*-cystine	*l*-tyrosine	*l*-tryptophane
BAW	0.36	0.078 to 0	0.9 to 0.83 and 0.014	0.33
BEAa	0.22	0.16 and 0	0.29 to 0.19	0.26
BEAb	0.32 and 0.22	0.19 and 0.042	0.23 and 0.18	0.44 to 0.41
2BA	0.33	0.25 and 0	0.22	0.31

times seen, but no other amino acids were detected. Several bands caused by unknown substances were encountered especially among the sera of patients with simple goiter.

DISCUSSION

Of the various factors studied chloride caused the most serious difficulty in chemical detection of iodinated compounds, and only a partial solution for the problem was found.

The mechanism by which chloride brings about reduction of ceric ion bears consideration. Direct reduction is known to occur but the quantity needed was considerably greater than the amounts detected. Sandell and Koltoff (2) showed that large amounts of chloride would act catalytically like iodide, but they further found that inclusion of chloride in the reagent greatly increased its sensitivity toward iodide. The presumption is therefore that this phenomenon permits the traces of iodine doubtless contaminating the reagents and the paper to cause bleaching of the area containing chloride at a faster rate than the slow bleaching on the background. Inclusion of chloride in the reagent obviated the difficulty caused by chloride and increased the sensitivity toward iodine, but the background color faded at an inconveniently rapid rate.

The thiouracil derivatives appeared to act catalytically in the same manner as iodide. It was clear that ceric ion was readily reduced by thiouracil, and thiouracil disulfide oxidized the arsenious ion in the strong acid used. The other substances which gave rise to spurious tests for iodine probably would not often give trouble because of the small quantities normally found in the serum, but medication with some of these compounds or with the antithyroid drugs might lead to difficulty.

Acknowledgment

We are indebted to Mrs. Esther Arra and Miss Stella Mothon for a great deal of assistance.

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FURTHER OBSERVATIONS CONCERNING THE ACTION OF PITUITARY THYROTROPIN ON THE INTERMEDIATE METABOLISM OF SHEEP THYROID TISSUE *in vitro*[1,2]

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ABSTRACT

Pituitary thyrotropin (TSH) significantly augments the assimilation of glucose by surviving sheep thyroid slices and promotes lipogenesis from, and oxidative decarboxylation of, glucose-U-C^{14} *in vitro*. TSH also enhances the incorporation of inositol-C^{14} into thyroid lipids even in glucose-free media. The observations support the general thesis that TSH acts by directly affecting the intermediate metabolism of thyroid tissue. Although the metabolic effects of TSH do not require exogenous substrate for *initiation*, the alterations in assimilative capacities may facilitate *sustained* TSH action upon thyroid function and growth.

ATTEMPTS to elucidate the mechanism of action of pituitary thyrotropin (TSH) have followed two directions. In the first, isotopic iodine has been employed to assess the phase of hormonogenesis which is primarily activated by TSH. Intrinsic to this approach has been the supposition that TSH disparately augments a single component of the biosynthetic sequence and that all other steps in iodine metabolism are activated secondarily. The alternate line of inquiry has been based on the premise that TSH affects more generalized facets of intrathyroidal metabolism, and that changes in iodine economy constitute merely one expression of these alterations. In this laboratory, the latter approach followed the demonstration that even the earliest step in the synthesis of thyroid hormone, i.e., the accumulation of ionic iodide against a concentration gradient, was dependent upon energy derived from intrathyroidal aerobic processes (1–3).

Received January 12, 1960.

[1] Presented in part at the Brook Lodge Symposium "Protein Hormones and Biologically-Active Peptides," Kalamazoo, Michigan, October 20–22, 1958 and at the annual meeting of the American Goiter Association, Chicago, Illinois, April 30–May 2, 1959.

[2] This investigation was supported in part by Research Grant A-1140 and Research Grant A-1571, National Institute of Arthritis and Metabolic Diseases, United States Public Health Service, Bethesda, Maryland.

[3] Investigator, Howard Hughes Medical Institute.

Accordingly, further studies into the effects of TSH upon the non-iodine metabolism of thyroid tissue were initiated (4) in the hope that "such investigations [might] disclose a unifocal, biochemical site of TSH action to which the other phenomena are related secondarily" (5).

The present report deals with the influence of TSH upon the assimilation of glucose and the disposition of glucose-U-C^{14} and inositol-C^{14} [4] by surviving sheep thyroid slices. *In vitro* effects of TSH upon all of these parameters are demonstrated. Thus, the studies support the thesis that the role of TSH must be viewed in terms of its generalized action upon intrathyroidal metabolic processes (5, 6). As in the past, the use of *in vitro* techniques was designed to facilitate temporal documentation and to eliminate the variable contribution of alterations in thyroid blood flow.

METHODS

Preparation of tissues and incubation: Thyroid glands were obtained from mature sheep following sacrifice by exsanguination in the abattoir. Glands were wrapped in cellophane and maintained at 10–15° C in chilled Dewar flasks during transport to the laboratory. Thyroids which had been excessively chilled, as evidenced by congealment of the adherent adipose tissue, were discarded. After freeing the thyroid of capsular and fascial investments, thin slices (0.3–0.4 mm.) were prepared with a Stadie-Riggs microtome. Slices were subjected to three serial rinses, of five minutes each, in 200 ml. of 0.150 M NaCl in order to remove all occluded extracellular fluids and to deplete intracellular metabolites by leaching. Thereafter, 250 ± 15 mg. quantities of slices were introduced into the main compartment of Warburg vessels. A torsion balance with an accuracy of ± 0.5 mg. was employed. All operations were performed at room temperatures.

The following suspending media were used for incubation: a) Modified Krebs-Ringer-Tris (KRT): 0.131 M NaCl, 0.005 M KCl, 0.0012 M $MgSO_4$, 0.008 M $CaCl_2$, and 0.010 M Tris (hydroxymethyl) aminomethane buffer (pH 7.4). This has been previously (5) designated as "phosphate-free" medium. b) Modified Krebs-Ringer-Phosphate (KRP): Electrolytes as in KRT save for the substitution of 0.010 M sodium phosphate buffer for the Tris (hydroxymethyl) aminomethane. This has been previously (5) designated as "phosphate" medium. c) Modified Krebs-Ringer-Bicarbonate (KRB): 0.120 M NaCl, 0.005 M KCl, 0.0012 M $MgSO_4$, 0.0008 M $CaCl_2$, 0.0012 M KH_2PO_4 and 0.025 M $NaHCO_3$.

In most studies, the *standard experimental procedure* described previously (5) was employed. Herein, 2.7 ml. KRT were placed in the main compartment of Warburg vessels, and side-arms were filled with 0.3 ml. KRP. In a few experiments, both side-arms and main compartments were filled with KRP or KRB. The side-arm solutions contained either 0 or 0.3 U.S.P. units of TSH ("Thytropar," Armour, Lot No. R 2402). This was prepared by dissolving weighed aliquots of the lyophilized TSH in appropriate media immediately prior to filling side-arms. Suspending media for most experiments were also supplemented with 100 units penicillin and 100 μg. streptomycin per ml. Control studies indicated that the inclusion of this quantity of antibiotics did not affect the metabolic functions of thyroid tissue which were under investigation.

For incubation by the *standard procedure* or in KRP, the vessels were gassed with 100% oxygen, and center-wells were filled with 5% KOH and fluted strips of filter paper.

[4] The term "inositol" will be used throughout the text to designate the naturally-occurring, physiologically-active isomer of hexahydroxycyclohexane which has been variously labeled with the prefixes *myo-*, *meso-* or *i-*.

The KRB studies were performed in an atmosphere of 95% O_2-5% CO_2, and no alkali was introduced into the center-wells. In all situations, following temperature equilibration at 38° C and gassing, the side-arms were tipped into the main compartment to yield a final concentration of 0.1 unit TSH per ml. in the "TSH-vessels" and to initiate timed observations. Thereafter, manometric readings were obtained at ten-minute intervals as described previously (1).

Substrates: To assess the disposition of exogenous substrates, media were supplemented with glucose ("Dextrose": National Bureau of Standards) or inositol ("i-inositol": Nutritional Biochemical Corporation) and, in some instances, with radioactive preparations of these compounds. The following radioactive preparations were employed:

a) Uniformly-labeled glucose, Glucose-U-C^{14}, Tracerlab, Boston, Mass. Lot #301-116-5.

b) Inositol-2-C^{14} (1.43 mg. labeled with 7.9 μc.) and inositol-U-C^{14} (7.15 mg. labeled with 9.8 μc.) were graciously provided by Dr. Laurens Anderson of Madison, Wisconsin. The inositol-U-C^{14} had been synthesized in the laboratory of Professor S. J. Angyal of New South Wales, Australia. Purity of the labeled glucose and inositol preparations was verified in this laboratory by paper chromatography in a) n-propanol/ethyl/acetate /water (24:13:7); b) phenol saturated with 0.1% aqueous NH_3 solution; and c) n-butanol/acetic acid/water (78:10:12), followed by radioautography of the chromatograms with No-Screen Xray. Concentration of radioactivity in the final suspending media ranged from 0.25 to 1.0 μc. of C^{14} per ml. The final glucose concentration was 2 mg./ml., and the concentration of inositol ranged from 0.25 to 0.36 mg./ml., a value equivalent to roughly one-tenth the concentration of free inositol in sheep thyroid tissue (7).

In a few experiments, the glucose-containing media were also supplemented with 1.7 mg./ml. of human serum albumin (Red-Cross H.S.A.) in order to evaluate the assimilation of glucose in the presence of glucose and albumin-complexed free fatty acids.

Analyses: Following incubation, the slices were quickly removed, blotted on filtepaper, reweighed on the torsion balance and frozen on solid CO_2. Center wells of Warr burg vessels were rinsed, filter paper strips were eluted with CO_2-free water, and the carbonate was quantitatively precipitated as $BaC^{14}O_3$, and plated on tared stainless steel planchets as described elsewhere (8). In experiments 1, 2, 3, and 15, total tissue lipids were prepared by homogenizing frozen slices in Potter-Elvehjem ground-glass homogenizers with 2:1 chloroform:methanol according to the method of Folch and Sloane-Stanley (9), and then washing extracts twice with 0.2 vol. of dilute salt solution (9) to remove water-soluble contaminants. For all other studies, frozen slices were homogenized with 5 ml. of chilled 5% TCA, and the centrifuged sediment was resuspended and washed two additional times at 4° C with 5 ml. portions of chilled 5% TCA preparatory to extracting lipids from the TCA-insoluble residue by the chloroform :methanol procedure as above. Measured portions of the washed chloroform: methanol extracts were plated directly on nickel-plated planchets at infinite thinness.

Duplicate aliquots of the media from vessels which had been incubated without tissues and from the experimental flasks were precipitated with $Ba(OH)_2$-$ZnSO_4$. Protein-free filtrates were analyzed for glucose with "Glucostat" reagents (Worthington Biochemical Corp., Freehold, New Jersey) by the enzymatic, colorimetric method of Huegett and Nixon (10).

Radioactive assay was performed with an automatic, micromil-window gas-flow counter (Nuclear, Chicago). $BaCO_3$ samples were corrected for self-absorption to a mass of 5 mg. at constant geometry. Sufficient counts were observed to reduce the probable error of the measurement to $\pm 3\%$.

Expression of results: For most studies, incubations were performed with duplicate vessels. In all instances, chemical analyses and planchetings were performed in duplicate.

Oxygen consumption was estimated in terms of QO_2 (mm^3 O_2 per hour per mg. of

initial wet weight). Net assimilation of glucose, expressed as "μg./mg. initial wet weight" was calculated on the basis of the difference in the final glucose concentrations in vessels incubated with and without tissues, and a known volume of 3.0 ml. of medium within each vessel. The disposition of radioactivity into $C^{14}O_2$ and lipid-C^{14} was quantitated on the basis of "% of total radioactivity per gm. initial wet weight." Total radioactivity was estimated with counting standards appropriately corrected to infinite thinness or to a mass of 5 mg.

RESULTS

1. *The Effect of TSH on Thyroid Carbohydrate Metabolism and Respiration in vitro:*

Results obtained in fourteen experiments with glucose-supplemented media are summarized in Table 1. QO_2 was measured in thirteen studies. Herein, TSH effected a uniform enhancement of oxygen consumption. Average QO_2 in the presence of 0.1 U.S.P. unit TSH/per ml. suspending medium was $18.7 \pm 3\%$[5] greater than control values, a significant effect ($P < 0.01$), and comparable in magnitude to the stimulation of thyroid respiratory metabolism by TSH which was previously observed with glucose-free systems (5). In twelve of the fourteen experiments, TSH promoted the assimilation of glucose from the suspending medium. Although TSH caused an occasional early increase in the hydration of suspended thyroid slices (5), the phenomenon could not be explained on this basis. For the fourteen experiments, the average net glucose "uptake" (as related to initial wet tissue weight) was $21.2 \pm 4.8\%$[5] greater in TSH-containing than in control vessels. TSH effects upon glucose assimilation were observed in media of low as well as high phosphate content (cf. "standard" or KRB vs. KRP media), and in simple saline-glucose systems as well as in media containing supplemental protein and free fatty acids[6] (cf. "standard" vs. "standard+HSA" media). Eight experiments were conducted with glucose-U-C^{14}. In all instances, lipogenesis was promoted by TSH. Average incorporation of radioactivity into thyroid lipids was $35.3 \pm 4.4\%$[5] greater in the presence of TSH. Effects upon oxidative decarboxylation of glucose were assessed by collecting CO_2 in seven experiments. Despite considerable differences in control respiratory activity in these studies, TSH invariably augmented the evolution of $C^{14}O_2$. The fraction of the total radioactivity which was recovered as $C^{14}O_2$ was $63.5 \pm 14.5\%$[5] greater in the TSH systems. It would seem unlikely that the action of TSH on carbohydrate metabolism can be attributed to contamination with other pituitary hor-

[5] Mean ± Standard Error of the Mean.

[6] That surviving sheep thyroid slices can actively metabolize albumin-complexed, free fatty acids (FFA) has been consistently demonstrated in the author's laboratory. Direct chemical analysis has been employed to document assimilation from glucose-free as well as "glucose-containing" media. Decarboxylation and incorporation into thyroid lipids *in vitro* have been shown with palmitate-1-C^{14}.

TABLE 1. THE EFFECTS OF TSH ON THE RESPIRATORY METABOLISM AND THE DISPOSITION OF GLUCOSE BY SURVIVING SHEEP THYROID SLICES

Expt. No.	Duration (hours)	Medium*	Control values†				+TSH: % of control values‡			
			QO_2	Glucose "uptake" (μg./mg.)	$C^{14}O_2$ (% added C^{14}/gm.)	Lipid-C^{14}	QO_2	Glucose "uptake"	$C^{14}O_2$	Lipid-C^{14}
1	4.0	Standard	0.24	4.4	7.33	1.18	125.0	115.9	165.1	145.8
2	4.0	Standard	0.25	4.4	6.86	0.97	128.0	118.2	166.0	131.9
3	4.0	Standard	0.54	5.7	17.65	1.93	103.7	135.1	123.6	144.0
4	3.0	Standard	0.29	3.8	5.69	0.49	117.2	126.3	140.6	142.8
5	3.0	Standard	0.37	4.5	9.32	0.64	110.8	115.5	122.8	106.3
6	3.5	Standard	0.34	3.0	—	—	111.8	110.0	—	—
7	3.0	Standard	0.62	5.2	—	—	114.5	88.5	—	—
8	4.5	Standard+HSA	0.58	7.3	—	—	117.2	131.5	—	—
9	4.5	Standard+HSA	0.40	4.9	—	—	135.0	124.5	—	—
10	3.0	Standard+HSA	0.44	3.5	—	—	102.3	100.0	—	—
11	2.0	Standard+HSA	0.47	3.4	—	—	125.5	123.5	—	—
12	3.2	KRP	0.21	1.8	1.57	0.20	114.3	166.7	222.3	132.5
13	3.2	KRP	0.37	4.0	5.02	0.36	137.8	115.0	203.8	135.6
14	3.3	KRB	—	4.2	—	0.25	—	126.2	—	139.2

* Medium: "Standard" refers to *standard experimental procedure* wherein 0.3 ml. of KRP were tipped-into main compartment of Warburg vessels containing 2.7 ml. KRT. In "Standard+HSA," these media were supplemented with 1.7 mg. human serum albumin per ml. Both side-arms and main compartments were filled with KRP or KRB in experiments so designated in Table 1. For details, see text. In all studies, except Expt. No. 6, 7, 10 and 11, the media also contained 100 units penicillin and 100 μg. streptomycin per ml. Following delivery of contents of side-arms, the reaction mixtures consisted of 3.0 ml. volume containing 2 mg. glucose per ml., and 0.3–1.0 μc. glucose-U-C^{14} per ml. in experiments 1–5, and 12–14.

† Absolute values for thyroid slices incubated without TSH in the suspending medium:

"QO_2": μl. O_2 per mg. initial wet weight during the first hour of incubation.
"Glucose Uptake": μg. glucose/mg. initial wet weight assimilated from the suspending medium.
"$C^{14}O_2$" and "Lipid-C^{14}": % of added radioactivity evolved as $C^{14}O_2$ or incorporated into lipids per gm. initial wet weight.

‡ Respiratory activity, assimilation of glucose, and disposition of glucose-U-C^{14} expressed as a percentage of control values. Added TSH consisted of 0.1 U.S.P. units per ml. final reaction mixture.

mones since comparable effects were not observed with equivalent quantities of bovine ACTH, porcine FSH, ovine prolactin or bovine STH.

For statistical analysis, paired t tests were performed on the differences of the logarithms of the absolute values observed in individual experiments. This is equivalent to testing whether the average ratio of values (i.e. +TSH/−TSH) is greater than unity (11). Results are summarized in Table 2. The effects of TSH on the assimilation of glucose and the disposition of glucose-U-C^{14} were thus shown to be highly significant ($P < 0.01$) in every case.

2. *The Effect of TSH on the Incorporation of Inositol-C^{14} into Thyroid Lipids:*

The studies with inositol-C^{14} were conducted in glucose-free media. Absence of detectable quantities of glucose was corroborated by enzymatic assay of the suspending media both before and after the incubation of sheep thyroid slices.[7] Nonetheless, in confirmation of earlier findings (5), TSH effected an average 18.2% increase of QO_2 above control values in the three experiments in which manometric readings were obtained (Table 3).

Previous observations had indicated that *in vivo* (5), as well as *in vitro*

[7] The author has been unable to observe measurable quantities of the enzyme glucose-6-phosphatase in sheep thyroid tissue. Presumably, therefore, any glucose in the suspending medium would have had to originate from reagent contamination or occlusion of body fluids in the slices.

TABLE 2. STATISTICAL ANALYSIS OF THE EFFECTS OF TSH ON THE DISPOSITION OF GLUCOSE BY SURVIVING SHEEP THYROID SLICES

	n*	Mean†	S.E.M.†	t	p
Glucose "uptake"	14	.0792	.0169	4.69	<0.01
$C^{14}O_2$	7	.2033	.0380	5.35	<0.01
Lipid-C^{14}	8	.1293	.0155	8.34	<0.01

* Number of experiments used for computation.
† Mean and standard error of the mean of the differences between the common logarithms of absolute values observed in the control and experimental vessels of individual experiments

(4), the turnover of P^{32} in the thyroidal phosphoinositide[8] exceeded that of any other phosphoglyceride within the thyroid. Results obtained with inositol-C^{14} in the present studies are summarized in Table 3. The incorporation of inositol-2-C^{14} as well as inositol-U-C^{14} into thyroid lipids was enhanced by TSH in all types of media. For the five experiments, the average increase above control values effected by TSH was $103.8 \pm 25.4\%$.[5] Paired t test of the differences of the common logarithms of values in individual experiments indicated that this stimulation was of statistical significance (Mean $\pm$S.E.M. of differences of logarithms $= 0.2943 \pm .0582$; $t = 5.06$; $p < 0.01$). More precise documentation of the disposition of radioactivity in individual experiments was precluded by the limited quantities of inositol-C^{14}. However, the lipid extracts which remained from all experiments after plancheting duplicate aliquots were pooled, and the distribution of radioactivity among individual thyroid phosphoglycerides was assessed by the mild alkaline hydrolysis and two-dimensional paper chromatography of Dawson (12), as described previously (4). By this technique, the only chromatographic spot which exhibited detactable radioactivity was the water-soluble hydrolysis product of phosphoinositide (i.e. the residue containing glycerol, phosphorus, and inositol (4)).

TABLE 3. THE EFFECT OF TSH ON THE INCORPORATION OF INOSITOL-C^{14} INTO THE LIPIDS OF SURVIVING SHEEP THYROID SLICES

Expt. No.	Duration (hours)	Type	Medium* volume (ml.)	Inositol† (mg./ml.)	Control values‡		+TSH: % of control values‡	
					QO_2	Lipid-C^{14} (% added C^{14}/gm.)	QO_2	Lipid-C^{14}
15	3.0	KRB	2.0	0.25	—	0.4510	—	142.3
16	3.3	KRB	3.0	0.33	—	0.0255	—	246.3
17	3.2	KRP	3.0	0.33	0.23	0.0525	130.4	142.1
18	3.0	Standard	3.0	0.36	0.34	0.0981	105.9	233.4
19	3.2	KRP	3.0	0.33	0.38	0.0339	118.4	254.9

* For definition of "type," see Table 1 and text. All media were supplemented with 100 units penicillin and 100 μg. streptomycin per ml. Experiment 15 was conducted with modified reaction vessel wherein 0.2 ml. were introduced from side-arm into 1.8 ml. contained in main compartment.
† Radioactive preparations were diluted with stable inositol to achieve desired concentration. Inositol-U-C^{14} (0.25 to 0.30 μc. per ml. final reaction mixture) was employed in experiments 15–17. Experiments 18 and 19 were conducted with inositol-2-C^{14} (0.29 and 0.26 μc. per ml. respectively).
‡ As in Table 1.

[8] The currently available information is based on the resolution of the glyceryl-phosphoryl residues (4) obtained following mild alkaline hydrolysis of lipid extracts (12). Thus, whether the thyroid phosphoinositide represents one compound or several substances differing in fatty acids or in P/inositol ratios cannot be said with certainty.

Net assimilation of inositol-C^{14} was vanishingly small. The release of preformed free inositol from the thyroid slices into the suspending medium was too great to assess whether TSH affected the assimilation of unlabeled inositol. The manifest leaching was not reduced by TSH.

DISCUSSION

In an earlier communication, it was reported that the action of TSH upon the respiratory metabolism of sheep thyroid slices could be demonstrated at the first manometric reading subsequent to the introduction of TSH—i.e., within ten minutes (5). Moreover, it was noted that the effects of TSH upon the incorporation of P^{32} into phospholipid phosphorus *in vitro* (13, 14, 5) could be extrapolated to zero-time to suggest virtually instantaneous activation (5). Comparable documentation of earliest temporal relationships was not attempted in the present studies. First, it was felt that demonstration of assimilative phenomena would require incubation of sufficient duration to effect concentration changes in the suspending medium which could be analyzed with accuracy. Secondly, it was felt that the magnitude of change in any single moiety need not parallel physiological significance. For example, an increased labeling of thyroid phospholipids by TSH *in vitro* must be preceded by an accelerated labeling of water-soluble phospholipid precursors. However, the intrathyroidal stores of precursors are small and in rapid turnover (4). Thus, the precursors attain early specific-activity equilibrium whereas the specific activities of the larger and more slowly turning over phospholipid pools continue to rise. Finally, evidence is mounting that the primary action (or actions) of TSH upon thyroid metabolism need not be reflected by the fate of any extracellular metabolite. For example, the stimulation of thyroid oxygen consumption by TSH *in vitro* (15–17) does not require the presence of any organic substrate in the suspending medium (5) and, in the absence of TSH, cannot be duplicated by supplementing the medium with glucose, pyruvate, lactate, nor any Krebs cycle intermediate (save possibly succinate) (18, 19). Furthermore, it was previously reported that TSH *in vitro* effected an increase in the intracellular concentration of inorganic phosphorus which could not be explained solely by an augmented penetration of extracellular orthophosphate (5). Since then, the author has obtained this effect even in "phosphate-free" media. Thus, it would appear that the quest for the primary site of TSH action might be directed most profitably towards an assessment of preformed intracellular stores of oxidative substrates (such as thyroidal fatty acids or glycogen), or upon the intracellular energy mechanisms, or enzymatic alignments which condition the disposition of these substrates.

It is against the above considerations that the present findings must be projected. Evidence has been presented that TSH profoundly alters the carbohydrate metabolism of surviving thyroid tissue. In the presence of

TSH, the accumulation of extracellular glucose by sheep thyroid slices is enhanced, and the evolution of $C^{14}O_2$ and the formation of lipid-C^{14} from glucose-U-C^{14} is promoted. The latter activities may be interrelated since oxidative decarboxylation of glucose via the thyroidal phosphogluconate oxidative pathway (20–23) would result in an increased availability of reduced triphosphopyridine nucleotide for lipogenesis (24, 25).[9] In addition, in glucose-free systems, it has been shown that TSH increases QO_2 and promotes the incorporation of inositol-C^{14} into phosphoinositide, thereby confirming that exogenous glucose is not required for *initiation* of the effects of TSH upon respiratory and phospholipid metabolism. The data do not justify attempts to reconstruct the progression of intrathyroidal biochemical events. Nor do they permit speculation concerning the mechanisms by which these metabolic phenomena are translated into an altered iodine economy (21). However, from the present studies, it may be inferred that the primary intrathyroidal sequence is accompanied by an augmented assimilative capacity for extrathyroidal glucose. Thus, regardless of endogenous metabolites, *prolonged* TSH action could be *sustained.* The over-all increase in glucose accumulation would result in an enhanced availability of co-factors and substrates for anabolic process whether TSH disparately activates the phosphogluconate pathway, as has been suggested in a preliminary report (22), or whether TSH does not alter the proportional disposition of glucose-6-phosphate along the alternative pathways for glycolysis within the thyroid.

Acknowledgments

The technical assistance of Miss Wilma Barshaw and Miss Harriet Provine and the secretarial services of Miss Susan Colby are gratefully acknowledged. The author is also indebted to Miss Margaret E. Drolette of the Harvard School of Public Health for her advice in the statistical analysis of the data. Sheep thyroid glands were obtained through the kind cooperation of Mr. Harold N. Larson of the New England Dressed Meat and Wool Co., Somerville, Mass.

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AVIAN NEUROHYPOPHYSIAL HORMONES: PHARMACOLOGICAL PROPERTIES AND TENTATIVE IDENTIFICATION[1]

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ABSTRACT

The pattern of pharmacological activities in the chicken neurohypophysis has been studied and described. These activities correspond to those of a mixture of oxytocin and arginine vasotocin, an analogue of oxytocin synthesized by Katsoyannis and du Vigneaud. Paper chromatographic and enzymatic studies, together with the demonstration of inactivation by thioglycollate, furnish additional evidence suggesting that oxytocin and arginine vasotocin may be neurohypophysial hormones in the chicken, *Gallus domesticus*.

PRESSOR and diuretic responses to the intravenous injection of chicken posterior pituitary extracts were originally demonstrated by Herring in 1908 (1). Mackenzie (2) found that such extracts also had potent galactogogue (milk-ejection) activity. Antidiuretic and oxytocic activities in extracts of fowl posterior pituitaries were first demonstrated by de Lawder, Tarr and Geiling (3), and Geiling subsequently reported that these activities were about one-half as strong as those present in U.S.P. Posterior Pituitary Powder (4). Frog water-balance activity was first described in the bird pituitary by Heller (5). Water-balance activity was far greater than in mammalian extracts of equivalent oxytocic and antidiuretic potency.

Detailed pharmacological and chemical studies of neurohypophysial hormones from vertebrates other than mammals have not been reported. In the present study we used manifold assays, chromatography and inactivation by trypsin and thioglycollate to characterize neurohypophysial hormones of the domestic chicken.

MATERIALS AND METHODS

Fowl extract. Posterior pituitaries were carefully dissected from freshly killed chickens (*Gallus domesticus*), dehydrated in acetone, desiccated *in vacuo*, and weighed. In the batch which was most thoroughly studied the average weight per dried lobe was 0.54 mg.

Received January 20, 1960.

[1] Supported by Research Grants Nos. H-1788 and A-1940 from the National Institutes of Health, U. S. Public Health Service and by a grant from the New York Heart Association.

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The dried lobes were ground in an agate mortar and extracted with a dilute solution of acetic acid, 0.154 M NaCl and 0.5% chlorobutanol at pH 3.0 in a boiling water bath for ten minutes. The resulting solution was filtered and placed in ampules which were sealed, immersed in boiling water for ten minutes and then refrigerated at 4° C until used for assay. Two other batches of fowl pituitaries were also prepared. The results of assays on these were not significantly different from those which will be reported.

Highly purified polypeptide hormones and analogues. These were kindly furnished by Prof. V. du Vigneaud and his colleagues of Cornell University Medical College. They were carefully desiccated and weighed in their laboratories and were then dissolved in the solvent used for the fowl extract, but without boiling. Each solution was then placed in ampules and treated in the same manner as the fowl extract.

Standards. The current U.S.P. Standard Posterior Pituitary Powder was used as reference standard throughout the experiments except where otherwise noted. Arginine vasopressin and oxytocin were used as standard in some assays. Each was carefully standardized against the U.S.P. Standard by appropriate pressor or rat oxytocic assay.

Biological assays:

a. *Rat Pressor.* Rats were anesthetized with urethane. Assay and statistical analysis of results were performed as recommended by the U.S.P. XV (6).

b. *Rat Oxytocic.* Two rat oxytocic assays were employed. The uterus was suspended in a van Dyke-Hastings solution without magnesium or in the same solution with 0.5 mM/l magnesium. The assay technique and statistical analysis of results were those of Holton (7) as modified by Munsick (8).

c. *Milk-Ejection.* Milk-ejection assays were performed on lactating rabbits by the method of Cross and Harris (9) as modified by van Dyke, Adamsons and Engel (10). A four-point design was used and the results were analyzed exactly as were those of the rat oxytocic assays.

d. *Dog Antidiurestic.* Intravenous antidiuretic assays were performed as described previously on hydrated, unanesthetized dogs (10). A four-point assay was used and the geometric mean potency is shown. No fiducial limits were calculated. The range of results in this assay is large and the reported potencies should be considered as approximations only.

e. *Rat Antidiuretic.* Intravenous antidiuretic assays in rats anesthetized with Inactin (sodium ethyl-(l-methyl-propyl) thiobarbiturate, Promonta) and ethanol were performed by the method of Jeffers, Livezy and Austin (11) as modified by Sawyer (12). The design and statistical treatment of results were similar to those used in the dog assays.

f. *Chicken Antidiuretic.* An intravenous antidiuretic assay in conscious chickens hydrated intravenously with hypotonic saline solution was utilized. Details of this assay will be published at a later date. Arginine vasopressin was used as the reference standard in these assays. Doses were given in a four point design and the geometric mean potency without fiducial limits was calculated.

g. *Hen Oxytocic.* An *in vitro* hen oxytocic assay was designed. Muscle strips of the uterine portion of the oviduct of laying hens were dissected and suspended in a van Dyke-Hastings solution containing 0.15 mM/l calcium, 0.5 mM/l magnesium and 100 mg. % glucose. The strips were 2–3 cm. long and 2–3 mm. wide. The reservoir and bath solutions were gassed with oxygen containing 5% carbon dioxide. The bath solution was maintained at a temperature of 43° C. These conditions were necessary to prevent spontaneous contractions. The assay was conducted exactly as described for the rat uterus. U.S.P. Standard was used for reference.

h. *Frog Bladder.* This *in vitro* assay was performed as recently described by Sawyer (13). Synthetic oxytocin (Syntocinon)[3] was the reference standard.

[3] We wish to thank Dr. R. Bircher of Sandoz Pharmaceuticals, Inc., Hanover, N. J., for a generous supply of Syntocinon.

TABLE 1. POTENCIES OF FOWL EXTRACT AND ARGININE VASOTOCIN IN U.S.P. UNITS/MG. ACCORDING TO VARIOUS METHODS OF ASSAY

Method of assay	Fowl extract		Arginine vasotocin	
	U./mg.	Limits*	U./mg.	Limits*
Rat pressor	0.75	0.74–0.77	64	62– 67
Rat ADH	0.77	G.M.	74	G.M.
Dog ADH	0.82	G.M.	51	G.M.
Rat oxytocic no Mg	0.92	0.85–0.98	37	34– 41
Rat oxytocic 0.5 mM./l. Mg	1.42	1.32–1.53	71	65– 78
Rabbit milk-ejection	1.45	1.41–1.48	79	75– 82
Avian depressor	2.0	1.3 –2.9	110	82–150
Turtle oxytocic	2.5	G.M.	220	G.M.
Hen oxytocic	7.3	G.M.	640	G.M.
Chicken ADH	12	G.M.	880	G.M.
Frog bladder	110	G.M.	10,000	G.M.

* 95% fiducial limits of potencies are indicated if applicable, otherwise potencies are geometric means (G.M.).

i. *Avian Depressor.* Conscious chickens restrained in the dorsal-recumbent, crucifix position were used in this assay. Local procaine anesthesia was used for arterial and venous cannulations. The usual four-point design was used, and statistical data were computed as in the rat oxytocic assay.

j. *Turtle Oxytocic.* The cloacal end of the oviduct of painted turtles (*Chrysemys picta*) which had ovarian follicles of 0.5–1.5 cm. diameter was suspended in a Frog Ringer solution (13). Assays were performed in the usual manner, using U.S.P. Standard for reference. The geometric mean potency is shown.

RESULTS

Pharmacological characteristics of avian neurohypophysial extract. Potencies of the extract of chicken posterior pituitaries in the above assays are shown in Table 1. All results are expressed as U./mg. of dried posterior lobes.

Significant discrepancies between avian depressor, milk-ejection and rat oxytocic potencies are evidence sufficient for the conclusion that the activities cannot be ascribed entirely to any combination of mammalian neurohypophysial hormones. The extremely high absolute and relative potencies in frog bladder, hen oxytocic, turtle oxytocic and chicken antidiuretic activities, coupled with relatively low pressor and rat oxytocic potencies, constitute evidence that there is at least one unknown active principle in the extract.

Mammalian neurohypophysial hormones and their analogues. An investigation of the structure-activity relationships of highly purified mammalian neurohypophysial hormones and their analogues was also being pursued in this laboratory (14). Each of these substances was compared with U.S.P. Standard in most of the assays previously mentioned (Table 2). Arginine vasotocin, synthesized by Katsoyannis and du Vigneaud (15, 16) contains high hen oxytocic, avian antidiuretic and frog bladder activities. No mean

TABLE 2. STRUCTURES AND ACTIVITIES OF NEUROHYPOPHYSIAL HORMONES AND RELATED SYNTHETIC ANALOGUES. POTENCIES ON EACH ASSAY ARE EXPRESSED AS U./MG. IN TERMS OF THE U.S.P. STANDARD. A, B, AND F ARE NATURAL PRODUCTS, THE REMAINDER ARE SYNTHETIC ANALOGUES

Structures

A.	*CyS-	Tyr-	Phe-	Glu(NH_2)-	Asp(NH_2)-	CyS-	Pro-	Arg-	Gly(NH_2)
B.			Phe				Lys		
C.			Phe				His		
D.			Ileu				Arg		
E.			Phe				Leu		
F.			Ileu				Leu		

Assays

Substance	Intravenous					*In vitro*			
						Oxytocic rat			
	Vasopressor, rat	Antidiuresis, dog	Anti-diuresis, fowl	Vasodepressor, fowl	Milk-ejection, rabbit	0.5 mM./l Mg	No Mg	Oxytocic fowl	Bladder permeability bull-frog
A. Arginine vasopressin	300	300	>300	42	51	15	9	240	c. 20
B. Lysine vasopressin	200	34		28	34	15?	5	15	< 5
C. Histidine vasopressin	2	9		5	18	7		<0.2	<12
D. Arginine vasotocin	60	50	880?	100	80	70	37	640	10,000
E. Oxypressin	3	6		55	88	22	16	<0.9	<20
F. Oxytocin	7	4	diphasic	360	360	360	420	29	360

* Position of the free amino group.

potency in terms of U.S.P. Units can be ascribed to arginine vasotocin or to the fowl extract in these three assays because variations in sensitivity between birds or frogs to the standard were more pronounced than to the fowl extract or to arginine vasotocin. The geometric mean potency may be close to the median potency in a population of animals and it is therefore used as an approximation.

Pharmacological Comparison of avian neurohypophysial extract to arginine vasotocin and oxytocin. Since the hen oxytocic and avian antidiuretic activities of arginine vasotocin are high, both relatively and absolutely, it was felt that this octapeptide might be a natural avian neurohypophysial hormone or closely related to one.

The pharmacological activities of arginine vasotocin and the fowl extract should be exactly parallel if the latter substance is the sole active hormone of the chicken posterior pituitary. The activities of the two are compared in Figure 1. It is apparent that there is a striking resemblance between them. Differences in dog and rat antidiuretic activities are not believed to be significant. There are, however, small but significant differences between the fowl extract and arginine vasotocin in rat oxytocic, milk-ejection and avian depressor potencies. The fowl extract is relatively more active than arginine vasotocin in each of these assays. This finding is consistent with the hypothesis that the fowl extract also contains an oxytocin-like hormone. In view of the other striking similarities to fowl extract it seems possible that arginine vasotocin might be the other active polypeptide. To ascertain further whether this could be true paper chro-

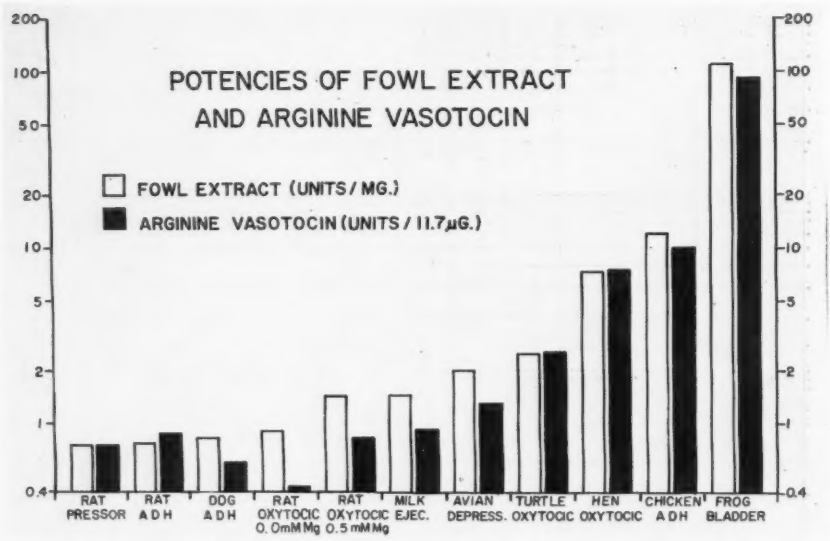

FIG. 1. Potencies of fowl extract and arginine vasotocin in 11 assays. Potencies plotted logarithmically, with scales adjusted to approximate pressor activities.

matography, enzymatic digestion and thioglycollate inactivation of the fowl extract were employed.

Paper chromatography. Heller and Lederis (17) showed that arginine vasopressin can be separated from oxytocin in crude posterior pituitary extracts by ascending paper chromatography. They found that the nature of the separated hormones can be tentatively designated by referral to the R_f's of purified hormone standards applied to the same paper.

We utilized this technique with several modifications. Highly purified oxytocin, lysine and arginine vasopressins and arginine vasotocin were employed as standards. Fowl extract was applied to the paper for comparison with these. Whatman No. 1 paper was used at all times in a *descending* system. The mobile phases used were either butanol:water:acetic acid (4:5:1) or butanol:water:acetic acid:pyridine (15:12:3:10). The former system was by far the more satisfactory, since there was less loss of activity and the spots were much more compact. Runs were usually made overnight for 16–22 hours at ambient temperatures. Staining of the spots was performed, as in Heller and Lederis' work, by the method of Reindel and Hoppe (18), utilizing orthotolidine. Elutions were made from macerated strips of the paper in 0.154 M NaCl at room temperature followed by centrifugation.

Figure 2 is a reconstruction of a chromatogram of the chicken extract, oxytocin, arginine vasotocin and lysine and arginine vasopressins. The

original chromatograms cannot be reproduced because the color developed by the Reindel-Hoppe method faded rapidly. It is apparent that the two vasopressins and arginine vasotocin have mobilities which are so propinquant that significant differences between them could not be detected by us. Oxytocin, on the other hand, moved far more rapidly, but it spread and "tailed" on all occasions. Although intense staining of the chicken extract occurred, the stains were scattered and did not correlate with biological activity. Two areas of rat oxytocic activity in the fowl extract were detected in this chromatogram. The majority of it was in the region common to the vasopressins and vasotocin but some of it was in the region occupied by oxytocin. All of the above observations were true of the butanol:water:acetic acid system as well as of the one shown. Recoveries of arginine vasopressin, as judged by pressor assays of the eluates from a pure sample and from the U.S.P. Standard, were 44 and 50% respectively. Recoveries of rat oxytocic activity from the fowl extract varied between 33 and 100%. Recoveries of oxytocin never exceeded 10%.

Failure to separate clearly arginine vasotocin from the vasopressins and to stain the active hormones in the chicken extract necessitated an alternative approach. The fowl extract was streaked in large quantities on the origin and runs were performed as before, using the butanol:water:acetic acid mobile phase. The entire area from origin to front was then eluted in various sized strips and the rat oxytocic potency of each eluate was determined. A typical elution experiment is illustrated in Figure 3. Again there was a distinct break between two areas of oxytocic activity, the proximal peak being compact and the distal one being diffuse. These eluates were

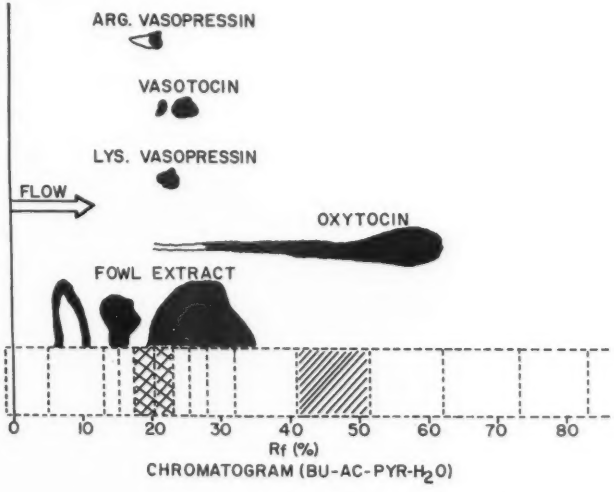

FIG. 2. Chromatogram of various pure polypeptides and fowl extract. Hatched areas indicate areas where rat oxytocic potency was found in eluates.

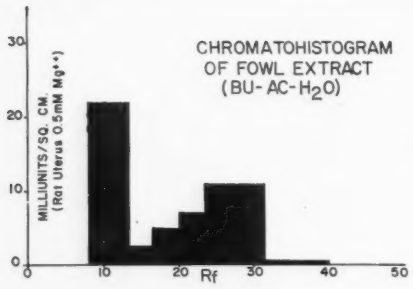

FIG. 3. Elutions from chromatogram of fowl extract, showing two peaks of activity.

then assayed on the hen uterus, and all activity resided in the proximal peak.

From one of these experiments two eluates were studied, one from the proximal peak and one from the center of the distal area. They were treated as separate unknowns, and pressor, rat oxytocic and milk-ejection assays were performed on both. The results are shown in Table 3. The proximal portion contained considerable pressor, rat oxytocic and milk-ejection activity. Its rat oxytocic potency was greater in the presence of magnesium than in its absence. The distal component contained no detectable pressor activity but did contain rat oxytocic and milk-ejection activities. Its rat oxytocic potency was not increased by the presence of magnesium. Oxytocin is the only neurohypophysial hormone or analogue studied which has equal or greater potency in the absence of magnesium than in a bath solution containing 0.5 mM/l. magnesium, when assayed against the U.S.P. Standard (8).

Tryptic digestion. Paper chromatography demonstrated the existence of two different hormones in the chicken extract, one resembling arginine vasotocin and the other resembling oxytocin. The proteolytic enzyme trypsin specifically cleaves peptide bonds only between the carboxyl group of arginine or lysine and the adjoining amino group of another amino acid. Trypsin has been shown to split glycinamide from arginine vasopressin. The heptapeptide which results is biologically inactive. Similar digestion of

TABLE 3. RELATIVE POTENCIES OF ELUATES FROM A CHROMATOGRAM OF FOWL EXTRACT
(All results are percentages of milk-ejection potency)

Fraction	Assay			
	Milk-ejection	Rat oxytocic*	Rat oxytocic†	Pressor
	Per cent	Per cent	Per cent	Per cent
Proximal	100	67	89	91
Distal	100	119	112	<22

* The bath fluid contained no magnesium.
† The bath fluid contained 0.5 mM./l. magnesium.

oxytocin does not significantly reduce its activity, since oxytocin contains neither arginine nor lysine (19).

Tryptic digestion was employed, therefore, to determine whether the fowl extract contains an active octapeptide with an arginine or lysine residue. If tryptic digestion destroys such a peptide, any residual activities must reside in a hormone that is, like oxytocin, unaffected by trypsin.

Fowl extract, oxytocin, arginine vasopressin and arginine vasotocin were all subjected to tryptic digestion with suitable controls. One part of each solution was diluted with two and one-half parts of a solution containing 0.5 mg./ml. of highly purified crystalline trypsin[4] in 0.154 M $NaHCO_3$, and one part was diluted with two and one-half parts of 0.154 M $NaHCO_3$ as a control. The pH's ranged from 7.74–8.39. After the incubation period the

TABLE 4. THE EFFECT OF TRYPTIC DIGESTION OF POLYPEPTIDES AND FOWL EXTRACTS
(All values are expressed as percentage of potency of controls)

Material	Assay			
	Pressor	Hen oxytocic	Rat oxytocic*	Rabbit milk-ejection
	Per cent	Per cent	Per cent	Per cent
Arginine Vasopressin	<1	<90	—	—
Arginine Vasotocin	<0.9	< 0.7	< 2	2
Oxytocin	—	—	80	80
Fowl Extract	<5	< 3	25	20

* The bath fluid contained no magnesium.

solutions were immediately refrigerated at 4° C until assayed. All assays were performed during the next 48 hours. The results are shown in Table 4.

The pressor activities of arginine vasopressin, arginine vasotocin and fowl extract were completely destroyed by tryptic digestion. Also the stimulating action of these three products on the fowl's uterus was abolished by tryptic digestion. Although no hen oxytocic activity could be demonstrated in the treated vasopressin, this particular uterus was extremely insensitive to the U.S.P. Standard, and we can therefore only state that there was less than 90% residual activity after tryptic digestion. On the other hand,about 80% of the oxytocin survived digestion as revealed by assays with the isolated rat uterus and with the lactating breast of the rabbit. Significant milk-ejection and rat oxytocic activity also remained in the chicken extract but these activities were absent in the digested arginine vasotocin.

These data indicate that the pressor and hen oxytocic component of fowl extract contains either arginine or lysine and that there is present in addition a hormone containing neither arginine nor lysine which has rat oxytocic and milk-ejection activities but very little pressor or hen oxytocic potency.

[4] No. TL 395-6 Worthington Biochemical Sales.

If one assumes that this oxytocin-like hormone is oxytocin and that it was inactivated under controlled conditions to the same extent as pure oxytocin one can compute that there are about 500 mU. of oxytocin per mg. of fowl posterior pituitary powder. This figure is based on milk-ejection activities which are probably more reliable than the rat oxytocic data because responses of the rat uterus were slightly inhibited by the presence of trypsin.

Of the eleven assays utilized only four will significantly reflect the contribution of oxytocin to the potency of the fowl extract. They are the rat

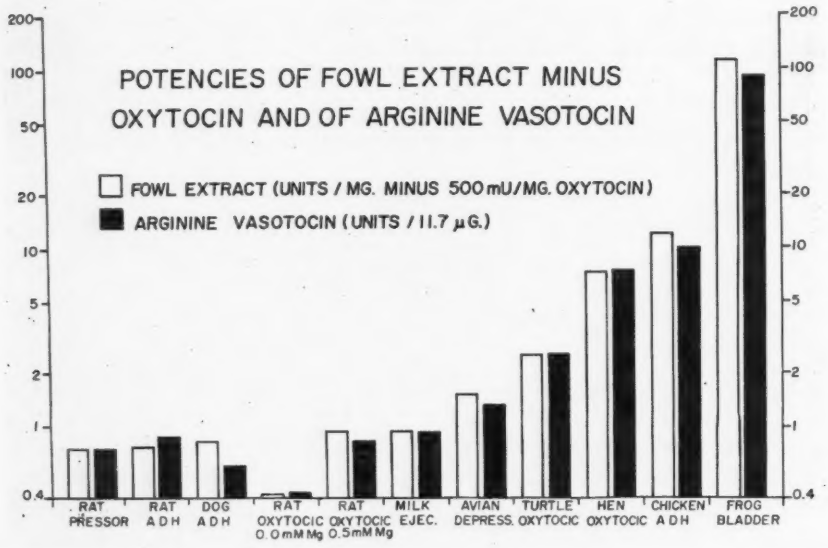

FIG. 4. Same as Figure 1, with 500 mU./mg. oxytocin deducted from potencies of fowl extract where oxytocin has significant effect (see text).

oxytocic with and without magnesium, the milk-ejection and the avian depressor assays. Five hundred milk-ejection mU. of oxytocin are equivalent to about 500 mU. in each of the other three assays. By subtracting 500 mU./mg. of fowl posterior pituitary from each of these four assays, the activity of the other hormone should be revealed as if it were assayed without oxytocin. This comparison is shown in Figure 4. No significant difference between fowl extract without oxytocin and arginine vasotocin exists in any of the eleven assays.

Thioglycollate inactivation. Thioglycollate inactivates mammalian neurohypophysial hormones (20). It was of interest, therefore, to determine whether the activities of fowl extract were also subject to such inactivation. The chicken extract, oxytocin and arginine and lysine vasopressins were

treated with 0.05 and 0.02 M sodium thioglycollate at pH 7.4, using controls of the same dilution at the same pH. All solutions were allowed to stand for 2 hours and were then assayed for pressor and rat oxytocic activities.

Pressor and rat oxytocic activities of oxytocin and arginine and lysine vasopressins were lost and no residual activity could be detected in the chicken extract after treatment with 0.05 M sodium thioglycollate. Thioglycollate in a concentration of 0.02 M caused nearly complete inactivation of each active substance. These results represent presumptive evidence that the avian hormones which cause oxytocic and pressor responses in rats probably contain a disulfide bond which had been reduced by thioglycollate.

DISCUSSION

The pharmacological and chromatographic characteristics of fowl posterior pituitary extract are consistent with the assumption that it contains oxytocin and another active peptide which is clearly neither arginine vasopressin nor lysine vasopressin. The activity of the second hormone is destroyed by trypsin. This implies that like the mammalian vasopressins this hormone contains an arginine or lysine residue. This peptide contains most of the hen oxytocic, hen antidiuretic, and frog-bladder activities of the extract.

Before arginine vasotocin had been assayed on the hen uterus the hen oxytocic potencies of other natural hormones and analogues had already been determined. Arginine in the 8-position of the side chain seemed necessary for high specific hen oxytocic activity, since lysine vasopressin, histidine vasopressin and oxypressin (leucine vasopressin) were far less potent than arginine vasopressin (Table 1). Isoleucine in the 3-position of the pentapeptide ring appeared to contribute to high specific hen oxytocic activity, since oxytocin was far more potent than oxypressin, which is identical with oxytocin except for the presence of phenylalanine in position 3. It was strongly suspected, therefore, that a compound with the oxytocin ring and the arginine vasopressin side chain would have higher hen oxytocic activity than the most potent nonapeptide known at that time, arginine vasopressin. In fact, arginine vasotocin contains activity of sufficient magnitude, relative to its pressor or oxytocic potency, to account for the response of the hen uterus to chicken extract. Arginine vasotocin also contains high relative hen antidiuretic, turtle oxytocic and frog bladder activities. These activities, as well as its chromatographic mobility and susceptibility to tryptic digestion, are all consistent with the assumption that arginine vasotocin is the other active polypeptide of chicken neurohypophysial extracts. However, positive identification of these active polypeptides must await their isolation in pure form together with confirmation of the structure of each (*See addendum added in proof*).

If we accept tentatively that the two hormones are arginine vasotocin and oxytocin, what are their functions in the chicken? Are they common to other birds? What hormones will be found in the neurohypophysial tissues of other vertebrate classes? Arginine vasotocin is probably the antidiuretic hormone of the chicken. It is more potent as an antidiuretic agent than arginine vasopressin in hypotonic saline diuresis. Further work is planned in this field. Oxytocin has a weak and inconsistent antidiuretic effect in enormous doses, but it is often diurectic.

The potent effect of arginine vasotocin on the uterine portion of the hen oviduct *in vitro* suggests an ecbolic function of this hormone in the hen. The intravenous injection of 30 rat pressor mU. of arginine vasotocin in an intact hen induced oviposition within ninety seconds. Intrauterine pressures were recorded once in a non-laying hen, and it was found that arginine vasotocin, arginine vasopressin and fowl extract caused equal, significant pressure changes in equivalent *in vitro* hen oxytocic doses. Oxytocin, in an equivalent *in vitro* hen oxytocic dose, did not affect the intrauterine pressure in this experiment. The required dose was so great that a marked vasodepressor and possibly other effects may have prevented the expected uterine response. Quantitative relationships among responses to these hormones on the hen uterus *in vivo* have not been established.

The other known effect of neurohypophysial hormones on the bird is the vasodepressor response. Since arginine vasotocin is much less potent than oxytocin in this assay, and since large doses of oxytocin must be administered to obtain such an effect, it seems doubtful that the response is of physiological importance.

From the quantitative data presented it appears that arginine vasotocin or a similar substance is present in a concentration of about 12 μg./mg. of dried fowl posterior pituitary. Oxytocin or a related compound, on the other hand, is far less abundant. Its concentration is about 1.2 μg./mg. These concentrations are quite different from those in the ox posterior pituitary where about 5 μg. of arginine vasopressin and 5 μg. of oxytocin are present per mg. of dried posterior lobe.

The function of oxytocin in the bird is still obscure. That its role may be that of a releasing factor for a hormone or hormones of the adenohypophysis is a distinct possibility although there is no evidence at present which can confirm or refute this hypothesis. We have not studied the neurohypophysial hormones of other birds. The data of Heller (5), when reviewed in terms of the experiments reported here, suggest that the pigeon also secretes arginine vasotocin.

Pickering and Heller (21) have independently confirmed our earlier report (22) of the existence of two active peptides in the chicken neurohypophysis. They have also separated chromatographically two similar principles in extracts from two teleosts and a frog. We have reported elsewhere on the close pharmacological resemblances between extracts from reptiles,

amphibians, teleosts and cyclostomes (23). Preliminary studies indicate that they also contain arginine vasotocin.

ADDENDUM

Since this paper was submitted Acher, R., J. Chauvet and M.-T. Lenci (*Biochim. Biophys. Acta* **38:** 344, 1960) have reported the isolation of oxytocin from fowl neurohypophysial extract and that it contains the same amino acids as mammalian oxytocin. Chauvet, J., M.-T. Lenci and R. Acher (*Biochim. Biophys. Acta* **38:** 571, 1960) also reported the isolation of two "vasopressins" from fowl extract to which horse "neurophysine" had been added to facilitate precipitation. "Vasopressin I" contains the amino acids characteristic of arginine vasotocin, thus they confirm our tentative identification of this peptide in the fowl neurohypophysis. "Vasopressin II," however, contains the amino acids of arginine vasopressin. This finding cannot be readily reconciled with our pharmacological and chromatographic studies which appear to rule out the presence of any appreciable quantity of arginine vasopressin.

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SELECTIVE INHIBITION OF THE CALORIGENIC ACTIVITIES OF CERTAIN THYROXINE ANALOGUES WITH CHRONIC THIOURACIL TREATMENT IN RATS

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ABSTRACT

Subcutaneous administration of thyroxine and some of its analogues to chronically thiouracilized rats revealed the following calorigenic potencies expressed as percentages of corresponding potencies in normal adult rats: L-thyroxine 7%; 3,5,3′-triiodo-L-thyronine, 3,5,3′,5′-tetraiodinated thyroacetic and thyropropionic acids, and a soluble thyroglobulin preparation, 29–43%; 3,5,3′-triiodinated thyroacetic and thyropropionic acids, about 100%. Further tests in normal and thyroidectomized adult rats, half of which received thiouracil, showed that the marked reduction in calorigenic effectiveness of thyroxine occurred in both types of the thiouracil-treated animals. The activity of triiodothyropropionic acid in thiouracilized rats was not significantly different from that observed in corresponding normal and thyroidectomized controls. The thiouracilized rats were found to be excreting, in their feces, approximately 2.3 times ($p < 0.01$) as much antigoitrogenically-active material as the nonthiouracilized rats, although both groups were receiving calorigenically equivalent doses of thyroxine which were in the ratio of 6:1 respectively. These studies suggest that thiouracil may inhibit utilization and may modify gastrointestinal excretion of thyroxine and some of its analogues. Lack of iodine in the 5′ position with absence of the amino group in the side chain of the analogues is associated with maximal calorigenic effectiveness when the animals are under the influence of thiouracil.

THE primary action of goitrogenic agents such as thiouracil is considered to be interference with the normal synthesis of hormones by the thyroid gland (1, 2). Specifically, these thiouracil-type compounds appear to prevent a peroxidase enzyme system in the thyroid gland from converting iodide to iodine *in vivo* (3). The results of several reports (4–11) suggest that such goitrogens may also selectively influence the pattern of peripheral action, degradation and excretion of thyroxine and triiodothyronine. The purpose of this report is to present new and confirmatory evidence of such action with several thyroxine analogues administered to rats maintained on thiouracil-containing diets.

MATERIALS AND METHODS

Groups of male albino rats, weighing 280–320 gm. each, were trained to eat their total daily requirement of food between 11:30 A.M. and 4:30 P.M. daily for seven to

Received June 5, 1960.

nine weeks prior to and during the test period. Some groups received powdered stock diet while others received thiouracil at a concentration of 0.1% by weight in the powdered stock diet. BMR tests consisted of three or six consecutive ten-minute readings of oxygen consumption for each rat on several days before, during and after administration of the calorigenic agents. These determinations were always made between 8:00 A.M. and 11:30 A.M. on rats in a post-absorptive state.

Thyroxine and one or more of the other compounds were usually assayed concomitantly at two or more dose levels per substance and five to twelve rats per dose level. Compounds were dissolved in a minimum volume of 0.1N NaOH and diluted to required concentrations with physiological saline. Daily doses for each rat were generally contained in a volume of 0.5–1.0 cc.

The method of evaluating calorigenic assays of this type was previously described in detail (12). The day to day BMR increments obtained at each dose level for the various compounds formed a series of parabolic curves when plotted on linear graph paper. A polar planimeter was used for computation of the total area under each curve. The two or more mean area-values derived for each compound were used to calculate final dose-response curves which became straight lines when Y was area and X was the logarithm of the dose. The resulting lines were so nearly parallel to the eye that they were redrawn on the basis of a common slope. The horizontal distances between any two lines, or their calculated X-intercepts, were then used for comparison of activities.

RESULTS AND DISCUSSION

At the beginning of these studies it was thought that essentially full-grown adult rats rendered hypothyroid by chronic administration of thiouracil would give a response to calorigenic agents similar to that of younger or surgically thyroidectomized animals. The inapplicability of this assumption became apparent even before the calorigens were injected. It will be seen from Table 1 that the pretreatment BMR of thiouracilized rats was not lower than that of normal controls having the same age and slightly heavier body weight. Actually, the oxygen consumption of thiouracil-treated rats tended to be higher. A similar observation was reported by

TABLE 1. OXYGEN CONSUMPTION OF ADULT MALE RATS MAINTAINED ON NORMAL OR THIOURACIL (0.1%) CONTAINING DIET FOR SEVEN TO NINE WEEKS

Assay No.	Diet	N	Av. mg. O_2/Kg./Hr.[a] ±S.E.[b]	Difference ±S.E. of diff.	P[c]
51	Normal	22	1333 ± 24	10 ± 34	>0.05
	Thiouracil	23	1343 ± 23		
61	Normal	24	1356 ± 23	137 ± 38	<0.01
	Thiouracil	24	1493 ± 30		
62	Normal	21	1349 ± 21	71 ± 39	>0.05
	Thiouracil	19	1420 ± 34		
Combined	Normal	67	1346 ± 13	74 ± 23	<0.01
	Thiouracil	66	1420 ± 18		

[a] Each rat was tested on four consecutive days to obtain its average BMR. These averages were then used to obtain the group average.

[b] S.E. of Average = $\sqrt{\Sigma d^2/N(N-1)}$.

[c] p-values based on Student's "t-test."

Petty and Martin (4) for the BMR of chicks fed 0.2% thiouracil diet. At the end of four months feeding, the chicks' BMR was 14% greater than that of the normal controls. The increased BMR was not further modified by an additional 9–15 days feeding of a 0.4% thiouracil diet. It was also observed (4) that normal and thiouracil-treated chicks responded equally well to the calorigenic effects of 2,4-dinitrophenol.

The thiouracilized rats (Table 1) ingested about 50 mg. of drug per kg. of body weight per day. In contrast, the daily maintenance dose for the clinical control of hyperthyroidism is of the order of 1–3 mg./kg. (13). The absence of BMR depression after chronic administration of thiouracil in rats (Table 1) and chicks (4) might be attributed to pharmacological side-effects resulting from high doses of the goitrogen. However, the appearance of the rats in this study and their definite gain in body weight did not suggest toxicity. Recently, Mellen (14) stated that conclusions regarding the metabolic effects of feeding thiouracil to young chickens were dependent on the length of fasting prior to determination of oxygen consumption. Compared with that of normals, the BMR of thiouracil-fed chicks gradually rose from about minus 11% (at 0–2 hrs. fast) to "normal levels" after about 24 hours. Since all of our calorigenic tests in rats were made after 17–19 hours of fasting, it appeared conceivable that the expected depressing effects of thiouracil on BMR, which were not observed after 17–19 hours, might have occurred earlier. However, in a control experiment comparing 24 normal and 24 chronically thiouracilized rats after 0–4 hours of fasting, the BMR of normals was as low or lower than that of the goitrogen-fed animals. Because body weights of the two groups were similar, calculations of BMR on a body surface area basis gave essentially the same results.

Calorigenesis after chronic thiouracil therapy:

Figure 1 illustrates the manner in which various thyroidally active compounds caused increased BMR in either the presence or the absence of thiouracil. L-Thyroxine, 3,5,3′-triiodo-L-thyronine, 3,5,3′,5′-tetraiodothyroacetic acid, 3,5,3′,5′-tetraiodothyropropionic acid and a soluble thyroglobulin preparation (hog) were considerably less active in the presence of thiouracil than in normal rats. On the other hand, the activities of 3,5,3′-triiodothyroacetic acid and 3,5,3′-triiodothyropropionic acid were not altered by thiouracil. Derivation of the X-intercepts for each of the parallel lines allows a quantitative interpretation of these results as shown in Table 2. The last column lists the relative activities of the compounds in thiouracilized versus normal rats. Thyroxine showed the greatest reduction in potency since the dose for equivalent calorigenesis was more than ten times greater in rats receiving thiouracil. The next four compounds listed had relative activities of about 29–43%. Finally, the activities of the triiodinated acetic and propionic acids appeared to be relatively little affected by

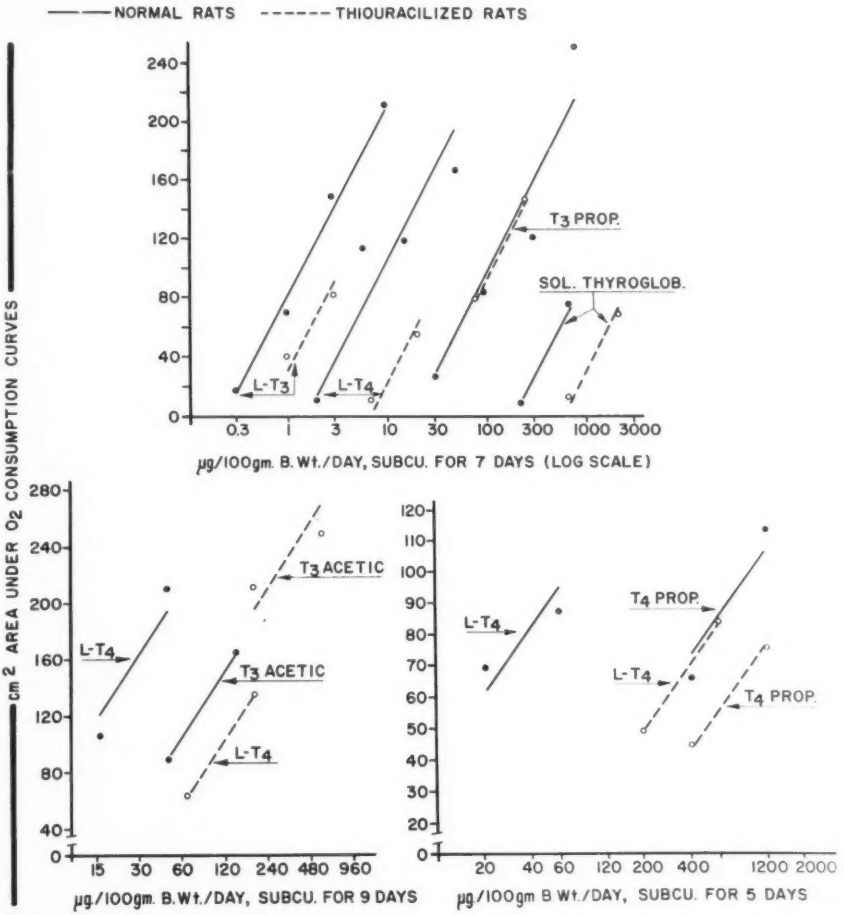

FIG. 1. *Thyroxine analogue induced calorigenesis in adult male rats maintained on normal or 0.1% thiouracil diet:* L-T3, 3,5,3′-triiodo-L-thyronine; L-T4, L-thyroxine (sodium pentahydrate); T3Prop., 3,5,3′-triiodothyropropionic acid; T3Acetic, 3,5,3′-triiodothyroacetic acid; T4Prop., 3,5,3′5′-tetraiodothyropropionic acid; Sol. Thyroglob., Soluble thyroglobulin (hog).

thiouracil. It is interesting to note that the X-intercept doses in the normal diet column show that only triiodothyronine (T3) was more potent than thyroxine (T4). However, in the thiouracil diet column similar comparison of doses indicates that the triiodinated acetic and propionic acids (Triac, Triprop) as well as triiodothyronine were more potent than thyroxine. Thus, T3 was about eight times more potent than T4 in rats on normal diet, as previously reported (12), but was nearly fifty times more potent in rats

receiving thiouracil. Triprop and Triac, although only 10 and 20% as potent as T4 in normally fed rats, were nearly twice as active as T4 in the thiouracilized animals.

From the last column of Table 2 it will be noted that thyroxine is the least effective compound. It has iodine in the 3,5,3′ and 5′ positions and an amino group in the side chain. In the group showing intermediate effectiveness, triiodothyronine has an amino group but lacks iodine in the 5′ position, while the tetraiodothyroacetic and propionic acids have a full complement of iodine but no amino group. Thyroglobulin is in the intermediate group probably as a result of its triiodothyronine content which, according to Mandl and Block (15), amounts to about 33% of the thyronine-bound iodine content (the remaining 67% is primarily in the form of thyroxine). On the basis of a thyroxine-triiodothyronine content in 2:1 ratio, thyroglobulin would be expected to have a relative effectiveness of about 20% in thiouracilized rats. The compounds which were the most effective (3,5,3′-

TABLE 2. THE CALORIGENIC ACTIVITIES OF VARIOUS THYROXINE ANALOGUES IN RATS MAINTAINED ON NORMAL OR THIOURACIL-CONTAINING DIETS

$$\text{(RELATIVE EFFECTIVENESS} = \frac{X\text{-INTERCEPT DOSE, NORMAL DIET}}{X\text{-INTERCEPT DOSE, THIO. DIET}} \times 100)$$

Preparation	Average X-intercept doses[a]				Relative (%) effectiveness with thiouracil
	Normal diet		Thiouracil diet[b]		
	No. of assays	X ± S.E.	No. of assays	X ± S.E.	
L-Thyroxine (sodium pentahydrate)	6	1.8 ±0.7	3	26.0 ±10.0	7
3,5,3′,5′-tetraiodothyroacetic acid	6	18.9 ±2.5	1	65.2	29
Soluble thyroglobulin (hog)[c]	1	191.9	1	595.4	32
3,5,3′,5′-tetraiodothyropropionic acid	1	38.0	1	96.7	39
3,5,3′-triiodo-L-thyronine	4	0.23±0.03	3	0.54± 0.24	43
3,5,3′-triiodothyroacetic acid	2	11.4 ±2.7	2	15.4 ± 2.4	74
3,5,3′-triiodothyropropionic acid	2	18.2 ±1.5	2	14.3 ± 5.3	127

[a] Calculated maximum μg. prep./100 gm. B. Wt./day/5–9 days, subcutaneously, which will not significantly increase the oxygen consumption of 300–370 gm. male rats.
[b] Thiouracil (0.1%) in diet fed for 7–9 weeks before and during administration of compounds.
[c] Soluble thyroglobulin: 0.77% total iodine; 0.21% thyronine-bound iodine (Blau, 1935).

triiodothyroacetic acid and 3,5,3′-triiodothyropropionic acid) lack iodine in the 5′ position and an amino group in the side chain.

It is generally accepted that the lack of iodine in the 5′ position of triiodothyronine imparts to this compound a special capacity for cellular penetration. Furthermore, the work of Gross (16) suggests that triiodothyronine (formed by deiodination of circulating thyroxine) is the substance which enters the cells and exerts the peripheral thyroid effect. However, our results indicate that in thiouracilized rats, triiodothyronine was very markedly prevented from exerting its effects on BMR (Table 2) even though it was far more effective than thyroxine. In contrast, the activities of the triiodothyroacetic and propionic acids, which may be regarded as deaminated analogues of triiodothyronine, appeared to be essentially unaffected by thiouracil. Crispell *et al.* (17) have stated that thyroxine ana-

logues possessing amino, carboxyl and phenolic groups compete with thyroxine for TBP binding sites and that the absence of any one of these groups markedly reduces the binding affinity. Thus, it appears conceivable that a deaminated side chain and lack of iodine in the 5′ position afford the optimal combination for calorigenic effectiveness of circulating thyroxine analogues in thiouracilized rats.

Influence of thiouracil on the actions of L-*thyroxine* (L-*T4*) *and 3,5,3′-triiodothyropropionic acid* (*T_3P*) *in normal and thyroidectomized rats:*

As shown above, the feeding of a diet containing thiouracil for seven to nine weeks did not produce, in intact rats, hypometabolic conditions similar to those expected after surgical thyroidectomy, as judged by the BMR before and after injection of calorigenic agents. Consequently, an experiment was designed to re-test L-T4, the least effective compound, and T3P, which was fully effective (Table 2), in thyroidectomized and intact animals with and without concomitant thiouracil feeding.

A total of 80 young adult male rats weighing 280–320 gm. each were used. Thirty were left intact and 50 were thyroidectomized and fed powdered rat diet, containing 1.0% calcium gluconate, *ad libitum*. After surgery, the usual outward signs appeared, i.e., loss of weight, lowered activity, evidence of lice and some mortality. Four to five weeks after operation, the 36 survivors and 30 intact males were placed in a special feeding apparatus for training on the daily fasting regimen. Half the rats in each group were fed diets containing thiouracil (0.1%) and calcium gluconate (1.0%) while the other half received diet containing only calcium gluconate (1.0%). These diets were continued until the end of the experiment. BMR standardization tests were started 12–13 weeks after operation (8 weeks after institution of special diets). On the basis of 8 daily BMR tests 12 rats from each of the 4 experimental groups were chosen and run in the final standardization series which consisted of 4 consecutive daily tests, as shown in Table 3. Thyroxine injections were started simultaneously, in all groups, at a level calculated to produce BMR increases in thyroidectomized but not normal rats.

TABLE 3. OXYGEN CONSUMPTION OF NORMAL AND THYROIDECTOMIZED ADULT MALE RATS AFTER 8 WEEKS FEEDING OF VARIOUS DIETS

Group	N	Diet	Av. gm. B. Wt. ±S.E.	Av. BMR* mg.O_2/Kg./Hr. ±S.E.
Normals	12	Powdered Rat Diet containing: 1.0% Calcium Gluconate	373 ± 12	1133 ± 29
Thyroidectomized	12	Powdered Rat Diet containing: 1.0% Calcium Gluconate	292 ± 11	1216 ± 38
Thiouracilized-Normals	12	Powdered Rat Diet containing: 1.0% Calcium Gluconate, 0.1% Thiouracil	368 ± 7	1140 ± 35
Thiouracilized-Thyroidectomized	12	Powdered Rat Diet containing: 1.0% Calcium Gluconate, 0.1% Thiouracil	268 ± 10	1167 ± 30

* See footnotes of Table 1.

As may be seen from Table 3, the net effect of thyroidectomy was apparent only in the body weight comparison where the average weight of thyroidectomized animals was about 25% less than the average weight of intact rats. There were no significant differences in oxygen consumption as a result of thyroidectomy and thiouracil treatment, separately or in combination. The four groups appeared to be statistically random samples from a homogeneous population with a mean oxygen consumption of 1164 mg. O_2/kg/hr. and an expected range of 1088–1240, at $p = 0.95$, for groups of 12 rats each.

The absence of BMR depression in the operated animals may be due to the fact that the oxygen consumption of the intact rats dropped 15 to 20% during the period between operation and BMR standardizations (compare Tables 1 and 3). On the basis of experience with thyroidectomized male rats, 70–80 days old and weighing 180 gm., a net reduction of about 25% in oxygen consumption is expected 6–8 weeks after operation, as compared with corresponding values in intact controls of the same age. Therefore, it appears that adult rats (under our maintenance conditions) may reach an athyroid level of metabolism equivalent to that of rats thyroidectomized after reaching adulthood. On the other hand, a recent publication (18) shows a high incidence of aberrant thyroid tissue in the thymus of Sprague-Dawley rats. After surgical thyroidectomy (and consequent increased TSH secretion) such tissue may be active enough to prevent the expected effect of thyroidectomy on BMR. Although the rats used in these studies were of the Wistar strain, there is a possibility that aberrant thyroid tissue was present in the thyroidectomized groups.

The BMR and body-weight changes resulting from gradually increasing thyroxine doses are shown in Figure 2. Percentage changes in the daily BMR for each group were calculated on the basis of a pretreatment value of 1164 mg. O_2/kg./hr. which was the pretreatment mean value for the 48 rats; zero per cent in Figure 2 corresponds to this level of oxygen consumption. During the first nineteen days of treatment the L-T4 dose was increased from 0.5 to 2.0 μg./100 gm./day. The intact thiouracilized rats responded like the normals, except for BMR increases of borderline significance at the 1.0 μg. dose level (Figure 2). On the other hand, both thyroidectomized groups responded significantly to L-T4, during the first 14 days, at dose levels in the range of those shown by Kroc and Stasilli (19) to be required to produce similar BMR changes in rats thyroidectomized at an earlier age. Of particular interest is the fact that the thyroidectomized rats returned to pretreatment metabolic levels in spite of continued and increasing thyroxine dosage between the 15th and 19th days. It will be seen that these rats gained an average of 32 gm. in body weight by the 19th day. It appears that the rapid weight gains may indicate a return to an essentially normal metabolic state. By the 15th day the BMR of these rats returned to pretreatment levels in spite of continued and higher daily dose of thyroxine. This phenomenon seems to be in agreement with the long as-

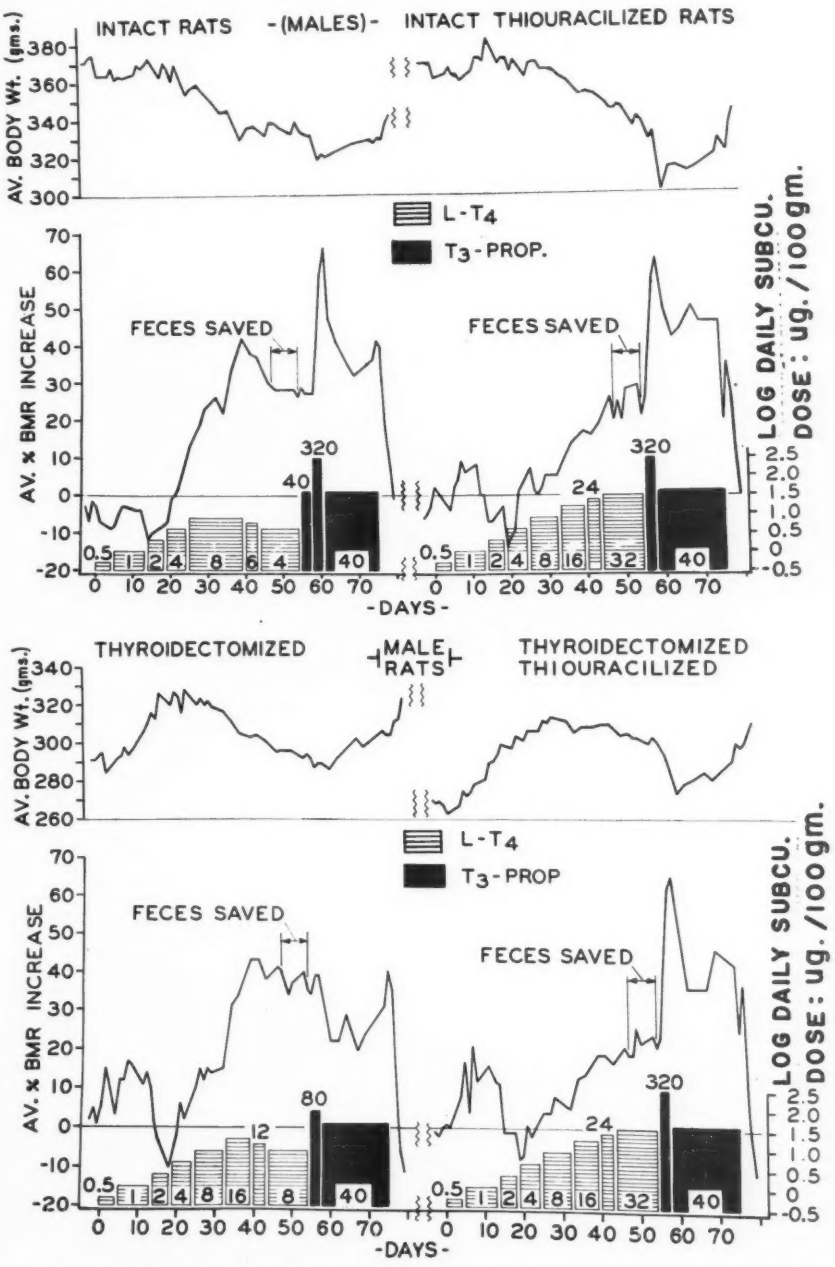

FIG. 2. *Effects of graded daily doses of* L-*thyroxine* (*L-T4*) *and 3,5,3'-triiodothyropropionic acid* (*T3Prop.*) *on BMR and body weight of adult male rats:* numerals within or just above dosage bars indicate μg./100 gm. body weight/day.

ymptote found in the response curves of thyroidectomized rats at physiological dose levels (19). The BMR in each group reached its lowest level during the period when all rats were receiving 2.0 μg. of L-T4 per 100 gm. /day. The agreement between the dose at this point in the BMR curves (at the 20th day) and the dose generally considered to be the L-T4 maintenance dose for rats in goiter-prevention assays is important. Subsequently higher dose levels were administered and, as expected, higher BMR values resulted.

Figure 2 also shows that in the presence of thiouracil, about eight times as much thyroxine was required as without thiouracil to induce an average BMR increase of about 30%. This agrees with the results of the more acute assays listed in Table 2. At these dose levels the body weights in the thiouracilized groups were much more resistant to the catabolic effects of thyroxine.

When T3P was substituted for L-T4, in calorigenically equivalent doses (40 and 80 μg.), no change in BMR was seen in the non-thiouracilized groups. However, similar substitution in the thiouracilized groups (32 μg. L-T4 substituted by 320 μg. T3P) produced highly significant increases in BMR and decreases in body weight. These results support the earlier observation (Table 2) that unlike L-T4, T3P is as active in thiouracilized rats as in animals on normal diet. Further support for this finding is shown by the identical response of the intact non-thiouracilized group to 320 μg. T3P.

After the highest T3P dose in each group a common dose of 40 μg. was substituted and responses were recorded for several days. In each group this dose appeared to be gradually cumulative. In the three days following the last injection, the BMR returned to Day 0 levels and significant increases in body weight were observed.

Goiter-prevention activities of feces from thyroxine-treated rats fed plain or thiouracil-containing diets:

During the last seven days of the ten-day period (Fig. 2, Days 45–55), when daily L-T4 doses were constant and the average BMR had plateaued, the feces were saved as indicated. The cumulative daily collections for each group were stored separately in a refrigerator prior to goiter-prevention activity assay. Pertinent data on body weight, L-T4 dose levels, BMR levels, and feces dry weight for the collection period are listed in Table 4. Preliminary tests with small samples indicated that the feces had to be combined, as shown in Table 4, in order to permit the use of a sufficient number of animals for statistical validity, in a 14-day goiter-prevention assay.

The two lots of feces were dried and pulverized through a 40-mesh screen in an electric mill. The feces from non-thiouracilized rats were designated feces A and those from the thiouracilized group, feces B. Diets containing 0.3% thiouracil and two concentrations of feces A or B were fed to female rats, weighing 160–190 gm., for a period of 14

TABLE 4. BODY WEIGHT AND FECAL EXCRETION OF INTACT AND THYROIDECTOMIZED ADULT MALE RATS (WITH AND WITHOUT THIOURACIL IN THE DIET) DURING A SEVEN DAY PERIOD WHEN DAILY DOSES OF THYROXINE WERE CONSTANT AND THE BMR HAD PLATEAUED[a]

Group	No. of rats	Av. gm. body wt.	μg. thyroxine per day subcut.			Av. % BMR increase			Dry weight[b] (gm.) of collected feces		
			Per 100 gm. body wt.	Av./rat	Av./ rat	Test Days	Per groups Av. ±S.E.	Per groups Av. ±S.E.	Total for groups Separate	Total for groups Combined	Av. excreted per rat per day
Intact	12	338	4	13.52	18.6[c]	5	28±1	32±2[c]	461	908	5.2[c]
Thyroidectomized	12	296	8	23.68		5	36±1		447		
Intact-Thiouracil Diet	12	343	32	109.76	103.5[c]	5	25±2	24±1[c]	446	850	
Thyroidectomized-Thiouracil Diet	12	304	32	97.28		5	23±1		404		

[a] See Figure 2 and text.
[b] Feces dried for 6 hours at 45° C lost an average of 30% moisture.
[c] Values to be used for comparative goiter-prevention assay results with feces (Table 5 and text).

days, *ad libitum*. Three control groups were also employed: one received only 0.3% thiouracil in the diet; another received a diet containing 0.3% thiouracil and 20% dried powdered feces obtained from normal adult male rats; and the third group (normal female controls) received normal powdered rat diet. A reference curve for determining the thyroxine-like activities of the feces was obtained by feeding additional groups of female rats 0.3% thiouracil diets containing three concentrations of thyroxine (Table 2). The thyroxine was added to the diet in the form of an alcoholic solution. This was followed by drying and mixing. At the end of 14 days on this feeding regimen, the rats were asphyxiated with CO_2 and their thyroids weighed. The results (Table 5) were analyzed in the manner described by Stasilli *et al.* (12, 20).

TABLE 5. GOITER-PREVENTION ASSAY OF FECES OBTAINED FROM THIOURACILIZED AND NON-THIOURACILIZED ADULT MALE RATS RECEIVING THYROXINE SUBCUTANEOUSLY. REFER TO TABLE 4 AND FIGURE 2

Bioassay diet group	Percentage concentrations in diet for 14 days[d]			No. of rats	Average thyroid gland weight mg./100 gm. B. Wt. ±S.E.
	Thiouracil	Feces	Thyroxine		
Thiouracil Control	0.3	0	0	5	19.7±0.8
Normal Feces[a]	0.3	20.00	0	3	19.1±2.4
Feces A—dose 1[b]	0.3	13.33	0	10	18.7±0.7
Feces A—dose 2	0.3	20.00	0	13	12.6±0.7
Feces B—dose 1[c]	0.3	13.33	0	10	10.5±0.7
Feces B—dose 2	0.3	20.00	0	13	6.0±0.3
L-Thyroxine Ref. Std. dose 1	0.3	0	0.000080	10	11.4±0.7
L-Thyroxine Ref. Std. dose 2	0.3	0	0.000120	9	10.0±1.0
L-Thyroxine Ref. Std. dose 3	0.3	0	0.000180	10	6.3±0.4
Normal Controls	0	0	0	5	6.0±0.7

[a] Control feces: from intact, untreated, normal adult male rats.
[b] Feces A: combined 7 day excrement of 12 intact and 12 thyroidectomized adult male rats eating powdered normal rat diet and receiving an average daily subcutaneous injection of 18.6 μg. thyroxine per rat. See Table 4.
[c] Feces B: same as feces A except rats were eating diet containing 0.1% thiouracil 14 weeks prior to and during daily subcutaneous injections of thyroxine averaging 103.5 μg. per rat. See Table 4.
[d] The ten groups of female rats ate an average of 11.5 gm./rat/day of the various diets (range for groups: 10.4–13.5 gm./rat/day).

From the data in Table 5 it was possible to calculate three curves with a common slope where the dose, X, represents the percentage concentration of feces or thyroxine in the diet and the response, Y, is the relative thyroid weight. From these curves the minimal doses necessary to maintain normal thyroid weights were derived. The equivalent dietary concentrations were: for feces A, 48.5%; feces B, 21.0%; L-T4, 0.000173%. On the basis of the average daily food consumption of 11.5 gm./rat/day, it was concluded that the biologically equivalent daily oral doses per rat were 5.6 gm. feces A, 2.4 gm. feces B and 19.9 μg. L-T4. The average daily fecal excretion of the A and B donors was 5.2 gm./rat (Table 4) and during the collection period the A donors were receiving 18.6 μg. L-T4/rat/day, subcutaneously, while the B donors required 103.5 μg./rat/day to maintain the same level of BMR stimulation. Therefore, the A donors, receiving normal diet, were excreting the equivalent of 18.5 μg. L-T4/rat/day, [(5.2/5.6)×19.9 μg.], while the B donors, receiving thiouracil in their diet, were excreting the equivalent of 43.1 μg. L-T4/rat/day, [(5.2/2.4)×19.9 μg.].

The difference between the thyroxine-like activities of feces A and B was statistically significant at $P = 0.01$. The rats which were under the influence of thiouracil required about six times as much L-T4 to maintain similar levels of increased oxygen consumption as did the rats eating normal diet. However, during the period of equivalent metabolic stimulation, the thiouracilized rats were excreting in their feces slightly more than two times as much thyroxine-like activity as the non-thiouracil group. It appears that the rats fed a normal diet excreted material(s) with activity equivalent to 99% of the injected dose while their thiouracilized counterparts excreted the equivalent of 42% of the injected dose. No conclusions can be drawn regarding the significantly different biological activities of the feces, because the nature of the thyroxine-like excretion products was not determined. However, in view of published reports of experiments concerned with the fate of radioactive thyroxine and triiodothyronine in normal, hypo- and hyperthyroid rats, and the results of our own studies, certain inferences may be considered.

In addition to the inhibition of thyroid hormone synthesis, thiouracil and related goitrogens apparently alter the rate of degradation and excretion of thyroxine in the rat, but the mechanism of this action is obscure and the evidence is somewhat contradictory. Thus, Kalant, Lee and Sellers (7) observed that the thyroid glands of propylthiouracil (PTU) treated rats trapped "appreciable" amounts of injected radioactive thyroxine and triiodothyronine while the glands of normal rats showed radioactivity "almost exclusively" in the inorganic iodide fraction. Hogness, Wong and Williams (6) and VanArsdel and Williams (10) found that PTU treatment caused an abnormal increase in the fecal excretion of radioactivity in rats injected with labeled thyroxine and triiodothyronine. The latter study

(10) suggested that PTU inhibited hormonal degradation "as represented by the low level of TCA-soluble (nonhormonal) radioactivity in the blood and by the smaller amounts of radioactivity excreted in the urine." Furthermore, the inhibited degradation of these analogues "seemed to be specific for the thiourea derivative since potassium perchlorate did not alter the rate of release of I^{131} from the labeled hormones to a TCA-soluble form" (10). In contrast to evidence for inhibition of hormone degradation, the studies of Ingbar and Freinkel (21) in patients with Graves' disease led these investigators to speculate that thyroid glands blocked by chronic goitrogen therapy may contribute to the degradation of thyroxine by secreting a product which stimulates peripheral deiodination, or by trapping and degrading the hormone within the blocked gland.

The decreased calorigenic effectiveness of thyroxine in thiouracilized rats suggests that the goitrogen may have inhibited the typical peripheral action and/or degradation of the injected hormone. At comparable levels of metabolic stimulation we observed that the excretion of thyroxine-like activities in the feces, relative to the injected doses, was far less in the goitrogen-treated rats. In contrast, other publications (6, 10) indicate that goitrogens increased the fecal excretion of hormone. This apparent discrepancy in the fecal excretion rates may be related to the difference in hormonal dose levels employed, e.g., physiologic levels in the radioactivity studies versus pharmacologic levels in our studies. However, in neither case was the nature of the excretion product(s) defined. It would be of interest to know how much of the radioactivity or biologic activity excreted in the feces was due to unchanged thyroxine, thyroxine glucuronide, lesser active degradation products of thyroxine and perhaps even triiodothyronine which, according to Gross (16), may be found in the feces after the administration of exogenous thyroxine.

Among other variables which may have influenced the results reported here are the enterohepatic circulation of thyroxine and other analogues as well as the known effects of thiouracil on intestinal flora and adrenals. The work of Taurog *et al.* (22) showed that as much as 75% of the radioactivity contained in an exogenous dose of thyroxine (ca. 80 μg.) was excreted primarily as intact hormone in the feces of normal rats in 48 hours. The relatively small amount of thyroxine glucuronide (T4-U) resulting from this dose was either reabsorbed in the small intestine or hydrolyzed by bacteria in the large intestine with release of free thyroxine. Therefore, no T4-U was found in the feces. Our rats, fed normal diet, were maintained at a level of 32% BMR increase with 18.6 μg. thyroxine per day (Table 4) and appeared to be excreting, in the feces, 99% of the daily dose. The latter rate appears to be considerably higher than that indicated by Taurog *et al.* (22) and could have been due to a trace of triiodothyronine resulting from the degradation of thyroxine and excreted in the feces (16). An excretion of only 1 μg. of triiodothyronine per day would account for the discrepancy. A plau-

sible explanation for the relatively lower rate of thyroxine-like fecal excretion (42% of the injected thyroxine activity) in the thiouracilized rats is not possible at present. The results of Taurog *et al.* (22) would appear to eliminate the possibility that the feces of goitrogen-treated rats contained relatively large amounts of biologically inactive T_4-U. However, the nature of the excretion products in thiouracil treated rats receiving thyroxine appears not to be established according to our knowledge of the literature. Perhaps altered liver metabolism produced by thiouracil may have increased T4-U secretion which might escape subsequent hydrolyses in the large intestine (22, 23). Recently, Flock and Bollman (24) have observed that congenitally jaundiced Gunn strain rats did not conjugate thyroxine and triiodothyronine with glucuronide, whereas glucuronide formation of the 3,5,3′-triiodinated acetic and propionic acid analogues was normal. Alteration of liver metabolism produced by chronic thiouracil treatment in our studies might explain the drastic reduction of calorigenic activity of thyroxine and other analogues in such rats as compared to the unaltered effectiveness of the 3,5,3′-triiodothyroacetic and propionic acids.

Alternatively, the lower percentage excretion of thyroxine-like activity in the thiouracilized groups may be related to the observations of Ingbar and Freinkel (21) which suggest that the goitrogen may increase peripheral deiodination of thyroxine and, as also suggested by Kalant, Lee and Sellers (7), that thyroid glands under the influence of goitrogen may trap exogenous thyroxine. A combination of such actions could result in relatively higher proportions of excretion products with little or no thyroxine-like activity in thiouracilized rats as compared to similarly treated rats fed normal diet.

That thiouracil inhibits the adrenals has been suggested by Money (8), and the direct effect of this goitrogen on these glands was demonstrated by Zarrow *et al.* (11). Direct physiological relationship between thyroid and adrenal activities are indicated by numerous reports, e.g., Menof (25), Swanson (26) and Akerman and Arons (27). Consequently, it is conceivable that in our studies, the observed differences between the calorigenic responses of normal and chronically thiouracilized rats to the various analogues may have been influenced by altered adrenal function in the presence of thiouracil.

Acknowledgments

Sam Reese, James Sapp and Larry Miller provided technical assistance while Frances Mackenzie and staff were responsible for animal care.

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NOTES AND COMMENTS

INCREASED MITOTIC ACTIVITY IN THE GLANDULAR STOMACH OF THE RAT FOLLOWING HYPOPHYSECTOMY

ABSTRACT

Eight Sprague-Dawley rats were hypophysectomized at 40 gm.; one was sham-operated. Sixteen days afterwards, 4 of the hypophysectomized animals were injected with STH (Somar, Armour), 20 μg. daily for 5 days. At this time, these animals and also 3 normal controls were labeled with 50 μc. thymidine H^3 and sacrificed 24 hrs. later. Integrated autoradiographs coated with liquid NTB^3 emulsion were exposed for 3 months. The number of H^3 positive cells in the glandular stomach was twice as large in the hypophysectomized group as compared to the controls and sham-operated. The growth hormone treated animals were comparable to the non-treated. The H^3 autoradiographic record was largely localized in all cases to the isthmus of the gastric glands.

The classical work of Leblond and Stevens (1, 2) has revealed a very fast renewal of the gastric and intestinal epithelium. Hypophysectomy reduces the mitotic rate in the intestinal mucosa (3); growth hormone restores it to normal (3). The effect of hypophysectomy on the mitotic rate of the stomach is still unknown. On the other hand, functional (4, 5) and structural (6, 7, 8, 9) defects have been produced by this operation. The present autoradiographic study is an attempt to observe the mitotic behavior of the glandular and non-glandular stomach in the absence of pituitary stimulation. It is based on the facts that H^3 labeled thymidine is specifically incorporated into DNA during preprophase (10) and that this nucleic acid is retained after routine histological procedure (11).

MATERIALS AND METHODS

Included in the present study were 8 Sprague-Dawley male rats hypophysectomized at 40 gm., one sham-operated animal and 3 controls. Sixteen days after hypophysectomy, four of the operated rats were injected with 20 μg. of growth hormone (Somar, Armour, Lot. No. R50109) for 5 days. After this treatment the controls and the sham-operated were labeled with 50 μc. H^3 thymidine (subcutaneous) and sacrificed 24 hrs. later.

The tissues were fixed in acetic-formaldehyde-ethanol (1:4:15), and cut at 10 μ in paraffin. Integrated autoradiographs (12) of H and E stained and unstained sections were prepared, utilizing liquid NTB^3 emulsion (Eastman Kodak).

Counts of H^3 positive cells were made at ×400 over circular fields with an area of 0.1017 $mm.^2$.

OBSERVATIONS

In the *fore-stomach*, the H^3 positive cells were localized in the stratum germinativum. The counts showed a 20% decrease two weeks after hypophysectomy and a slight (10%) increase after 5 days of growth hormone treatment (Table 1).

In the *fundus* of controls and sham-operated animals, labeled cells were limited to the area of the isthmus of the glands (Fig. 1). In the hypophysectomized (Fig. 2) and growth hormone treated rats, they were more widespread. The counts in controls, (Table

TABLE 1. H^3 THYMIDINE-LABELED CELLS PER FIELD (0.1 $MM.^2$)

Groups	Fore-stomach			Fundus		
	Rats	Field	H^3+ Cells	Rats	Fields	H^3+ Cells
Controls	3	30	13.2 (±0.06)	3	30	24.6 (±0.12)
Sham operated				1	10	28.4
Hypophysectomized	3	30	10.5 (±0.06)	4	40	53.6 (±0.6)
Growth normone	4	40	11.7 (±0.27)	4	40	52.9 (±0.1)

Received November 12, 1959.

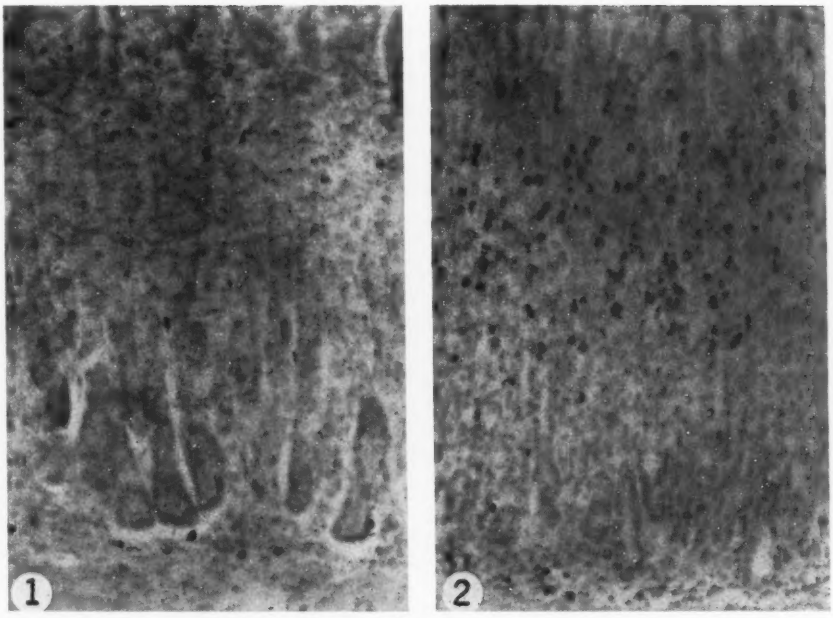

FIG. 1. Fundus, control. Integrated pre-stained autoradiograph, ×155.
FIG. 2. Fundus, hypophysectomized rat. Integrated pre-stained autoradiograph, ×155.

1) revealed approximately twice as many H^3 positive cells in this area as compared to the fore-stomach. On the other hand, both hypophysectomized and growth hormone treated rats and about twice as many H^3 positive cells in the fundus as compared to the controls and sham-operated.

When observed against an H and E background, the H^3 positive cells appeared to have been distributed at random over a wide portion of the gland (Fig. 2). However, when the autoradiographic record was compared to a PAS stained section, it became evident that most of the H^3 positive cells were located above the mucous neck cells and consequently belonged to an elongated isthmus, the cells of which had maintained their mitotic potential (2).

It was already known that in the absence of a sufficient pituitary stimulation, the pepsinogenic and acidogenic cells of the glandular stomach undergo secretory atrophy (4, 5, 6, 7, 8, 9). It now appears that this condition is accompanied by hypertrophy of the isthmus with increased karyokinesis.

Acknowledgment

The author is indebted to Mrs. L. F. Bélanger for skilled technique, to Eastman Kodak Co. (Rochester, N.Y.) for providing NTB^3 Emulsion and to the Medical Division of National Research Council of Canada for a grant-in-aid.

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THE ACCUMULATION OF FLUOROBORATE IONS IN THYROID GLANDS OF RATS[1]

ABSTRACT

The distribution of fluoroborate ions in rats was investigated using F^{18} as tracer. It was found that this ion accumulates specifically in the thyroid gland, showing a behavior similar to iodide ions. Perchlorate and iodide ions were found to interfere with fluoroborate uptake. The results corroborate former assumptions on the mode of action of perchlorate and its isoelectronic ions on the iodide trapping mechanism of the gland.

The effect of fluoroborate ions (BF_4^-) on the iodine uptake in the thyroid gland of rats was found similar to that of perchlorate(ClO_4^-). ClO_4^- ions were shown to accumulate in the thyroid to a higher extent than in other tissues(2). The thyroid blood concentration gradients of ClO_4^- attained at doses of 5–20 mg. $KClO_4$ were comparable to the gradients found for I^- at the same doses; however, these values were much lower than those for tracer doses of I^-. The purpose of the present study was to determine whether the analogy between BF_4^- and ClO_4^- ions may be further extended, by following the distribution of BF^{4-} in various tissues, particularly in the thyroid gland. As BF^{4-} ions labelled with F^{18} are available at much higher specific activities than $Cl^{31}O_4^-$, it became possible to investigate their behavior at μg. doses and to compare it with that of I^- ions. It should be kept in mind that the plasma iodine level is also in the μg./100 range.

METHODS

Carrier-free F^{18} was prepared by bombarding targets of $Li_2CO_3{}^{18}$ (90–95% O^{18}) by 3.1 Mev protons in a Van de Graaf electrostatic accelerator (3, 4). The carrier-free F^- ions were equilibrated with BF_4^- by isotopic exchange in acid solution (4). The labeled BF_4^- was then neutralized and the excess F^{18-} was removed by isotopic dilution with CaF_2. As the rate law of $BF_4^- - F^-$ isotopic exchange is first order in HF and in free H^+ (4), there was no further exchange in neutral solution. It has been further proved that neither hydrolysis nor isotopic exchange of BF_4^- take place to any detectable extent at 37° C. in saline solution at pH = 7, buffered by phosphate buffer.

Solutions of KBE, labeled with F^{18} were injected intraperitoneally into adult rats (avg.

Received November 16, 1959.

[1] This work has been partially supported by a grant of the Fund for Promotion of Research from the General Federation of Labor in Israel.

wt. 200 gm.) at various dose levels. After a given interval, the rats were sacrificed and various tissues removed, weighed and counted in a NaI well type scintillation counter. Special care was taken to separate the thyroid glands from the surrounding tissues, as the cartilage of trachea was found to accumulate F^- ions selectively. The extent of accumulation of BF_4^- in tissues was expressed in terms of specific tissue activity. Specific activity (counts/min./mg. carrier) was calculated after normalizing the activity to a standard zero time, to correct for the radioactive decay. Knowing the specific activity of the BF_4^{-18}, it is easy to derive the amount of BF_4^- concentrated per one gm. tissue. The specific activities of the various tissues were related to that of blood (2); the values presented in the experimental results are relative specific activities.

The distribution of carrier-free F^- ions was determined by an analogous procedure. In some experiments $KClO_4$ or NaI were added to the injected KBF_4 labeled with F^{18}.

RESULTS AND DISCUSSION

The distribution of BF_4^- in various tissues of rats is presented in Table 1. The distribution of F^- in a number of tissues was determined for comparison. Each value is an average of determinations in four rats. It is evident from these results that the only tissue that accumulates BF_4^- to an appreciable extent is the thyroid gland. The results with F^- ions show that the thyroid tissue has no affinity for F^-. The selective accumulation of F^- in the cartilaginous rings of the trachea is of significance. This rise in activity, which may be due either to fast uptake or to isotopic exchange, should be kept in mind when following the behavior of F^- in the vicinity of the thyroid gland.

The effect of BF_4^- dose on the concentration gradients of this ion in the gland, as well as its rate of uptake, is demonstrated in Table 2. It is shown that increasing the dose of BF_4^- from μg.'s to mg.'s decreases the concentration gradient by a factor of 20 or more, to values well within the range of ClO_4^- or I^- concentration gradients observed in the mg. range(2). On the other hand, the BF_4^- concentration gradient in the μg. range is comparable to that attained with tracer doses of I^- in glands temporarily blocked by propylthiouracil (1). The similarity between the behavior of BF_4^- and I^- ions is

TABLE 1. THE DISTRIBUTION OF BF_4^- AND F^- IN VARIOUS TISSUES OF RATS*
(Tissue/blood sp. activity ratio)

Dose	5 μg. KBF_4	5 μg. KBF_4	5 μg. KBF_4	5 μg. KBF_4	C.F.†LiF^{18}
Time after injection (min.)	40	60	100	120	120
Tissue					
Muscle	1.93	1.65	1.90	1.42	—
Liver	1.27	1.34	0.99	1.24	0.15
Spleen	1.35	1.01	0.85	1.03	—
Brain	0.67	0.23	0.49	0.86	0.21
Thyroid	12.3	15.7	21.0	36.4	0.23
Bone (Tibia)	—	—	—	—	112.0
Cartilage of Trachea	—	—	—	—	261.0

* Four rats per group.
† C.F.—Carrier-free.

TABLE 2. THE ACCUMULATION OF BF_4^- IN THYROIDS OF RATS*
(Thyroid/blood sp. activity ratio)

Time after injection (min.)	60	120	210	300
Dose (μg. KBF_4)				
5	12.0	21.0	42.0	61.0
50	—	13.4	13.6	—
500	—	—	12.0	—
1000	1.75	—	2.3	—
5+5000 μg. $KClO_4$	7.1	—	—	—
5+1000 μg. NaI	3.04	3.83	—	—

* Four rats per group.

further manifested by the effect of ClO_4^- and I^- ions on the BF_4^- accumulation. These results are analogous to former findings (1) on the effects of ClO_4^- and BF_4^- as inhibitors of the iodine uptake. As has been pointed out elsewhere (2), thiocyanate ions interfere with thyroid function by some other mechanism.

The similarity in the behavior of I^- and ClO_4^-, which was discussed elsewhere (2), could not be followed into the range of tracer doses. The behavior of the BF_4^- ion, which is isoelectronic with ClO_4^-, shows a similarity to iodide down to the tracer level.

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THE EFFECT OF PARATHYROID EXTRACT UPON TRANSAMINASE ACTIVITY IN METAPHYSEAL BONE

ABSTRACT

Comparisons were made of the glutamic-oxalacetic transaminase (GOT) activity of metaphyseal segments of the tibias of parathyroid-treated rats with those of controls. GOT activity was found to be significantly increased, in relation either to the dry weight of the tissue or to its N-content. It is concluded that parathyroid hormone exerts a marked influence on protein metabolism at the transaminase step, and that this may be related to the resorption of bone.

Recent papers have illustrated a trend toward attributing the actions of certain hormones to their effects upon enzyme activity. Gavosto *et al.* (1) have suggested that cortisone increases gluconeogenesis by enhancing transaminase processes. Rosen *et al.* (2), shortly thereafter, added the proposal that the control of hepatic levels of glutamic-pyruvic transaminase (GPT) by glucocorticosteroids is related to the mechanism whereby these compounds exert their gluconeogenic activity. Laskin and Engel (3) found that oxygen consumption of bone slices was diminished following administration of parathyroid extract, and attributed this effect in part to a depression of the succinic dehydrogenase system; they also proposed a theory of bone resorption which depends upon the accumulation of intermediary metabolites, resulting from a decrease in their oxidation, owing to the effect upon the dehydrogenating enzymes. Zuchlewski and Gaebler (4) studied the effects of growth hormone on L-glutamic acid dehydrogenase (GAD), and the transaminases, GPT and glutamic-oxalacetic transaminase (GOT), in hypophysectomized rats. The author (5, 6) has found that transaminase activity, both of GOT and of GPT, is present in the zones of endochondral ossification of long bones, being more active in the zone of the secondary spongiosa, where resorption is especially prominent.

Received December 29, 1959.

and has suggested a possible connection between transamination and resorption of bone. As a further test of this proposal, transaminase activity has been studied in the metaphyseal bone of animals treated with parathyroid extract.

Twenty Sprague-Dawley male albino rats of an average weight of 150 gm. were divided into 2 groups of 10 animals each. Of the 2 groups, kept on a standard diet, 1 received a daily subcutaneous injection of 100 U.S.P. units of parathyroid extract (Paratormolo, Ism Milano) for each animal, while the second group was used as controls, and received daily injections of physiologic salt solution. Treatment was discontinued on the sixth day, and the animals were killed on the seventh day, 12 hours following the final dose of parathyroid extract; during the final period the animals were fasted. The proximal half of each tibia was collected and chilled in ice, and the metaphysis and the upper half of the shaft were separated from the epiphysis and the marrow was removed. The segments so obtained were used for the determination of GOT activity; this was carried out on homogenates (high speed blendor homogenization followed by filtration through gauze) using α-ketoglutarate as substrate and aspartate as

TABLE 1. GLUTAMIC-OXALACETIC TRANSAMINASE (GOT) ACTIVITY OF RATS' TIBIAS (METAPHYSIS AND UPPER HALF OF DIAPHYSIS)

	Control rats	Parathyroid-treated rats
μmol oxalacetate/100 mg. dry wt./10 minutes	23 ± 3.1*	49 ± 5.8*
μmol oxalacetate/1 mg. N/10 minutes	4.6	7.9

* Average values of 10 determinations $\pm$S.E.

$-NH_2$ donor, following the technique of Tonhazy *et al.* (7). The final colorimetric readings were made with a DU Beckman spectrophotometer. Nitrogen determinations were made on samples digested with sulfuric acid, after Johnson (8).

A comparison of the values found in the segments of the tibias of animals treated with parathyroid extract, and those from the control animals, demonstrates a significant difference in GOT activity between the two groups (Table 1).

The increased transaminase activity found in the bones of parathyroid-treated rats, in a region where histologic examination reveals intense osteoclastic and resorbing activity, seems to support the view of a participation of the enzyme, and of the metabolic steps controlled by it, in the process of bone resorption. This finding is in agreement with the previous observation (5, 6) of high transaminase activity in the sites of bone growth and remodeling. That the values for increased enzyme activity, based on the dry weight of the bone segments studied, do not reflect simply a relative increase in the organic matrix fraction is shown by the significantly higher values for GOT activity in the bones from the parathyroid-treated animals when the activity observed is related to the nitrogen content of the homogenates (Table 1).

Although this study does not establish the true relationships between resorption of bone, osteoclastic activity, transamination, and the parathyroids, the coincidence of elevated transaminase activity with increased resorption of bone seems to mark a step forward in the understanding of these relationships. The parathyroid hormone exerts a marked influence on protein metabolism at the transaminase step. Whether this deviation is related to demineralization, either as one of the causes or one of the consequences, has not been shown, and will be the object of further investigation.

So far as the action of parathyroid extract on the organic matrix is concerned, Talmage and Elliott (9) suggest that it elicits metabolic processes in bone enabling calcium to be dissolved in concentrations greater than can be accounted for by the solubility of apatite crystals, and Striganova (10) has observed that parathyroid extract raised the

proteolytic activity of bone tissue of both rats and rabbits. As it has been shown that decreased transaminase activity generally accompanies protein anabolism (4, 11), and that the reverse is true (1), the results here reported would indicate, in agreement with Striganova, that a protein catabolic process is parallel to the parathyroid-induced resorption of bone. The high transaminase activity, leading to local gluconeogenesis, might be one of the ways through which the parathyroid hormone raises the citrate level in bone and contributes to the solubilization of the bone mineral (12). Supporting this view is also the decreased serum amino acid level observed in man (13) after administration of parathyroid extract.

Whether the increased transaminase activity is to be referred to the osteoclasts themselves, since they are a prominent feature of the resorption of bone induced by parathyroid extract, or to the protein of the matrix, is a problem which might be solved by the aid of histochemical methods for transaminase; this problem awaits the development of suitable methods.

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Acknowledgment

The author is grateful to Dr. Franklin C. McLean for his assistance with the preparation of the manuscript.

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DUCTO-ALVEOLAR GROWTH IN MAMMARY GLANDS OF ADRENO-GONADECTOMIZED MALE RATS BEARING MAMMOTROPIC PITUITARY TUMORS[1]

ABSTRACT

The hormonal product(s) of grafted mammotropic pituitary tumors (MtT) induced extensive ducto-alveolar growth in mammary glands of adreno-gonadectomized male rats receiving no other hormones. Cortisol, but not desoxycorticosterone trimethylacetate at doses used, "permitted" milk secretion in mammary glands of MtT-bearing, adreno-gonadectomized rats; it did not stimulate mammary gland growth.

Experiments in laboratory animals with purified preparations from pituitary glands of other species suggest that a combination, including adrenal and ovarian steroids as well as hypophyseal hormones, is essential for ductoalveolar development of the mammary glands (1, 2). In contrast, experiments in which grafted mammotropic pituitary tumors (MtT) have been used as an endogenous source of mammotropic hormone(s) (MtH) have led to the postulate that the secretory products of the pituitary mammotropes (Mt) are the primary stimulant of mammary growth. In the latter experiments, steroid hormones seemed to play modifying roles; e.g. similar ducto-alveolar growth occurred in the mammary glands of otherwise untreated intact or gonadectomized MtT-bearing rats of either sex and in estrogen-treated, hypophysectomized MtT hosts (3–5). General promotion of growth was consistently associated with the mammary gland stimulating effect (4).

Growth of the mammary glands of adreno-gonadectomized, MtT-bearing rats has been observed, but in experiments previously reported, such animals had received supportive doses of adrenocorticoids (5). This communication reports the extensive ducto-alveolar development induced by isologous MtH in adrenogonadectomized MtT-bearing male rats receiving no other hormones. Milk secretion occurred when cortisol, but not desoxycorticosterone trimethylacetate (DOC-TMA), was administered to such rats.

MATERIALS AND METHODS

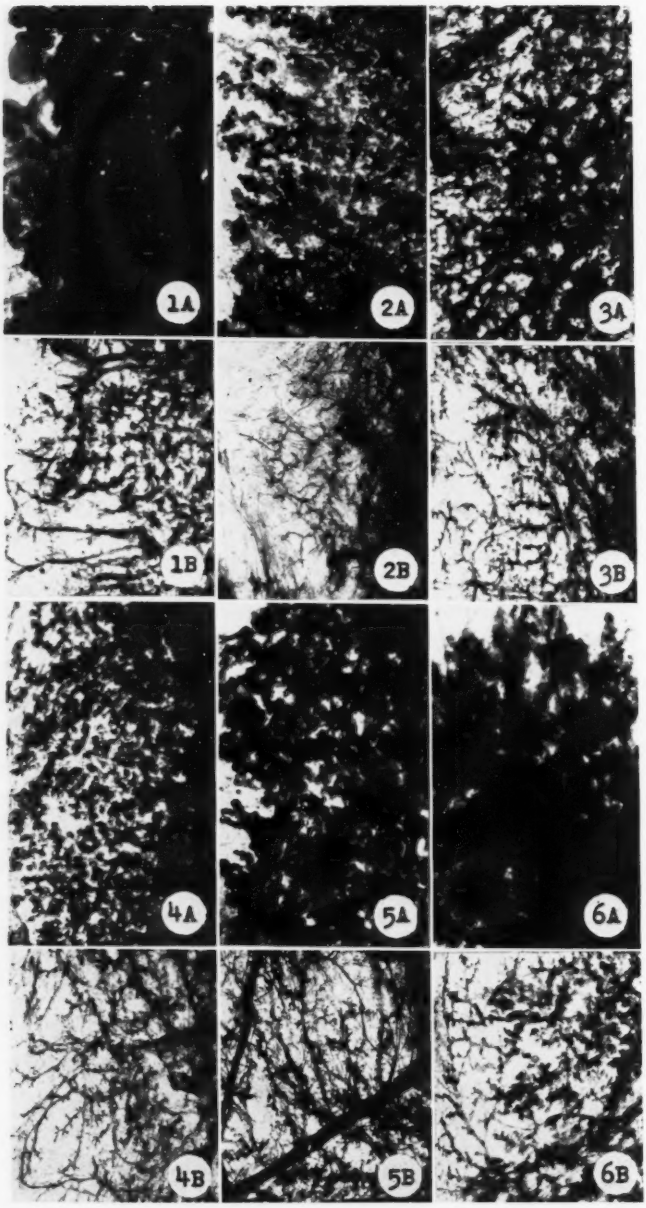
Forty-five male rats of the Fischer (F) strain were adreno-gonadectomized and four to sixteen days later 31 of these were grafted intramuscularly in the left thigh with MtT strain F4 (sub-passage XXIIIb) (3, 4). Nine F males were gonadectomized and six of these grafted with MtT at the same time. The animals, which were 3 to 5 months old at the time of grafting, were distributed as evenly as possible according to age throughout the treatment groups. Steroid hormones were administered from the day of grafting of adreno-gonadectomized animals according to the following regime:

Six MtT-bearing and two control rats received one subcutaneous injection of 2.5 mg. DOC-TMA per week.
Six MtT-bearing and three control rats received one injection of 12.5 mg. DOC-TMA per week.
Six MtT-bearing and three control rats received three subcutaneous injections of 1.0 mg. cortisol in aqueous suspension per week.
Seven MtT-bearing and three control rats received three injections of 5.0 mg. cortisol per week.

No corticoids were administered to six adreno-gonadectomized MtT-bearing, three adreno-gonadectomized control and to the nine gonadectomized rats. The diet of Rockland rat pellets was supplemented with vegetables and bread and milk daily. Adreno-gonadectomized rats receiving steroids were given the choice of 1% saline or tap water *ad libitum*. Those receiving no steroids were given saline only.

At autopsy 40 days after grafting one inguinal fat pad containing the mammary gland was dissected free, spread, and fixed in 10% formalin. Spread glands were coded, dehydrated, weighed, stained with hematoxylin, dehydrated and stored in mineral oil. Ducto-alveolar development of the stained coded glands were rated on an arbitrary [illegible]

Received January 12, 1960.
[1] Supported by grants from the National Cancer Institute.



Note: Figures 1 through 6 are photographs of whole mounts of mammary glands from MtT-bearing male rats (indicated by "A") and their respective controls (indicated by "B"). Final weights of grafted MtT and surgical and hormonal treatment and the rating of development and secretory activity in the mammary glands are given for each animal. All photos enlarged 4.5 times.

scale, and the degree of secretory activity on a 0 to 3^+ scale. Mammary gland area was estimated from measurements of two dimensions with a millimeter rule.

RESULTS

As in previous experiments (3–5), mammary glands of gonadectomized male rats bearing MtT were composed of nearly confluent masses of alveoli and ducts distended with secretion (Figs. 1A and 7). Those of gonadectomized males without MtT consisted of branched ducts with few alveoli and no secretion (Fig. 1B). Extensive alveolar development occurred in adreno-gonadectomized MtT-bearing rats in the absence of steroid replacement (Figs. 2A and 7) although milk secretion was minimal and the area and defatted weight were moderately reduced (Fig. 7). Treatment of adreno-gonadectomized MtT-bearing rats with either DOC-TMA or cortisol had little effect on the degree of ducto-alveolar development and but slightly increased mammary gland weight (Figs. 3A, 4A, 5A and 7). Secretion was markedly increased by the higher dose of cortisol, however (Fig. 6A).

Mammary glands of adreno-gonadectomized rats without MtT or steroid replacement were rudimentary (Fig. 2B). At the doses used, cortisol maintained mammary ducts and alveoli of such rats at near the castrate level (Figs. 5B, 6B and 7). DOC-TMA was somewhat less effective (Figs. 3B, 4B and 7).

The higher dose of cortisol suppressed the average per cent increase in body weight of MtT-bearing adreno-gonadectomized rats (Fig. 7). The gain in weight of all other groups of adreno-gonadectomized MtT-bearing animals equaled or exceeded that of the gona-

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FIG. 1A. 5.3 gm. MtT; gonadectomized; no steroid hormone therapy. Note the confluent mass of alveoli greatly swollen with secretion. Rating: 4^+ development, 3^+ secretion.

FIG. 1B. No MtT, otherwise as in Figure 1A. Note branched ducts with few alveoli. Rating: 1^+ development, 0 secretion.

FIG. 2A. 3.2 gm. MtT; adreno-gonadectomized; no steroid hormone therapy. Note extensive alveolar development throughout. Rating: 3^+ development, 0 secretion.

FIG. 2B. No MtT, otherwise as in Figure 2A. Note rudimentary branched ducts. Rating: 0.5^+ development, 0 secretion.

FIG. 3A. 0.7 gm. MtT; adreno-gonadectomized; 2.5 mg. DOC-TMA per week. Note thickened branched ducts bearing small alveoli. Margin of inguinal lymph node appears at right edge of picture. Rating: 2^+ development, 0 secretion.

FIG. 3B. No MtT, otherwise as in Figure 3A. Note rudimentary brached ducts. Rating: 0.5^+ development, 0 secretion.

FIG. 4A. 1.9 gm. MtT; adreno-gonadectomized; 12.5 mg. DOC-TMA per week. Note extensive alveolar development throughout. Rating: 2^+ development, 0.5^+ secretion.

FIG. 4B. No MtT, otherwise as in Figure 4A. Note rudimentary branched ducts with few tiny alveoli. Rating: 0.5^+ development, 0 secretion.

FIG. 5A. 3.9 gm. MtT; adreno-gonadectomized; 1.0 mg. cortisol three times per week. Note extensive alveolar development and moderate secretion. Rating: 3^+ development, 0.5^+ secretion.

Fig. 5B. No MtT, otherwise as in Figure 5A. Note rudimentary ducts with few tiny alveoli. Three blood vessels are shown. Rating: 1^+ development, 0 secretion.

FIG. 6A. 1.1 gm. MtT; adreno-gonadectomized; 5.0 mg. cortisol three times per week. Note confluent mass of alveoli swollen with secretion. Rating: 3^+ development, 2^+ secretion.

FIG. 6B. No MtT, otherwise as in Figure 6A. Note branched ducts with small alveoli some of which contain secretion. Rating: 1^+ development, 0–0.5^+ secretion.

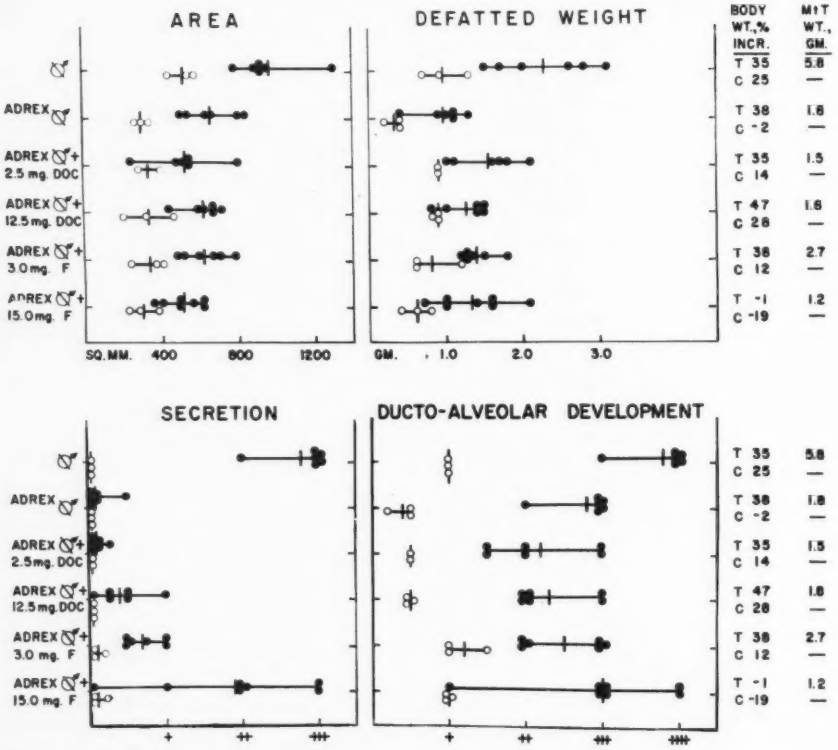

FIG. 7. Effect of MtT on mammary glands of gonadectomized and adreno-gonadectomized male rats treated as indicated in left hand column. Solid circles indicate individual values from rats bearing MtT, and open circles, those from their respective controls. Group means are indicated by vertical cross lines. Figures in right hand columns are averages of the respective tumor-bearing (T) and control (C) groups.

dectomized tumor hosts, however, despite the fact that final tumor weights were lower in the doubly operated animals (Fig. 7). Of the adreno-gonadectomized rats without MtT, the percentage increase in body weight was greatest in animals receiving 12.5 mg. DOC-TMA per week.

DISCUSSION

The results support the hypothesis that the hormone(s) secreted by Mt are the main stimulants of ducto-alveolar growth of the mammary gland of young adult rats and that steroid hormones are not essential for such growth. Previous studies have indicated that estrogen acts as a stimulant of the Mt, and in hypophysectomized animals, has little or no mammary growth-promoting action (3–5). In agreement with others (1, 2), however, a glucocorticoid was necessary for milk secretion. The higher dose of cortisol was most effective in this respect even though it inhibited growth in general. A cortisol dose between those used here might prove optimal for both body growth and milk secretion and corticosterone, the principal product of the rat adrenal cortex (6) might be more effective.

The relationship between the hormone(s) released by Mt and those purified from pituitary glands of other species is not yet clear. Promotion of body growth and stimulation of the mammary gland have thus far occurred in parallel in MtT hosts of a given treatment group (4). Homogenates (7) and extracts (8) of MtT tissue stimulate the pigeon crop gland and homogenates induce growth of dwarf mice[1] and moderately increase the width of the tibial plates of hypophysectomized rats[2] (7). Assays of MtT tissue preparations for follicle-stimulating and luteinizing (7), luteotropic,[3] thyrotropic (8), and adrenocorticotropic (9) hormones have been negative, in confirmation of observations in host animals (3–5).

Failure to induce marked ducto-alveolar development in rats with purified heterologous growth hormone and/or prolactin in the absence of steroid hormones could be due either to species differences in hormone structure and the consequent antigenicity, or to difficulty in maintaining high circulating hormone levels by injection. In contrast, the hormone(s) secreted by MtT are native to the rat and are presumably released in steadily increasing quantities during tumor growth.

Acknowledgment

The authors are indebted to Dr. John W. Anderson for the photographs.

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INHIBITION AND SYNCHRONIZATION OF MATING IN THE MOUSE BY ORAL ADMINISTRATION OF PROGESTINS

ABSTRACT

Diets containing either progesterone or 6α-methyl-17α-hydroxyprogesterone acetate were fed to 95 adult female mice. Mating was inhibited in all cases. Inhibition was effective after a preliminary treatment period of 4 days and extended beyond end of treatment for at least 64 hours. Of the 80 mice which did mate, 71 mated 64–72 hours after normal diet was resumed and the 9 others mated 24 hours later. Eighty-nine per cent of the mice which mated were pregnant when autopsied 12 days after mating. The pregnant females had a mean of 10.4 living fetuses each. Ingestion of treated diets by males did not affect their fertility or libido.

Reliable control of estrous cycles by some relatively simple procedure would be useful in certain techniques, such as ova transfer and artificial insemination, in both laboratory animals and larger domestic animals. It is well established that continued injection of progesterone to animals with normal estrous cycles will inhibit estrus and ovulation and that upon withdrawing treatment, normal estrous cycles are resumed. This technique has been used by many workers (1) in an attempt to synchronize estrus and ovulation in groups of domestic animals.

The inconvenience of making daily injections to large numbers of animals of readily absorbed and metabolized materials and the lack of a sharp withdrawal point of slowly absorbed or metabolized materials has limited the usefulness of this approach. Orally administered steroids have recently been shown to be effective in inhibiting mating (2) and as a substitute for endogenous progesterone in maintaining pregnancy (3). The following experiments were conducted to test whether administration of progestins via the diet would inhibit mating in mice and whether mating would be synchronized in a group of mice following simultaneous withdrawal of treatment.

MATERIALS AND METHODS

Laboratory bred, albino mice kept under normal daylight conditions were used in this study. Most of the females were adults which had produced at least one litter and most of the males were known to be fertile.

Crystalline progesterone and 6α-methyl-17α-hydroxyprogesterone acetate[1] (U8839) were each tested. Progesterone or U8839 were added to the diet at the rate of 6 mg. and 0.2 mg. per gm. of dry feed respectively.

The mice were fed normal laboratory diet (Rockland) or one of the treated diets and water *ad libitum*. The treated diets were prepared by the following procedure. The normal pelleted diet was ground; the progestin was dissolved in 95% ethyl alcohol and sprayed on the ground feed which was then thoroughly mixed. Water or reconstituted dry skim milk was added to the treated diet at the rate of 1 cc. to 2 gm. of diet. This was again mixed and formed into $\frac{1}{2}'' \times \frac{1}{2}''$ pellets by a crude hand-operated device or formed into pressed patties about $\frac{1}{2}''$ thick and 2″ in diameter. The reformed diet was air dried in a 65° C. oven for about 24 hours. The dry patties were broken into smaller pieces to permit feeding in normal cage feeders.

Feed and feeders were weighed daily and the wasted feed recovered under the cages was also weighed to determine the actual amount of feed eaten daily.

Females were caged and fed treated diets in 12 separate groups of 7–10 mice per cage for at least 4 days prior to putting each individual female with a male in his cage. Shorter preliminary feeding intervals were not tested. This preliminary feeding regime was instituted to give the progestins time to be effective before females were exposed to males. Treatment was started without regard for the stage of the estrous cycle of the females. Females were fed a treated diet for periods ranging from 4 to 15 days. In some cases normal diet was restored to females when transferred to the male's cage; in other cases females continued receiving a treated diet for 7 or 10 days after pairing (Table 1). In all cases the male received the same diet as his mate while they were paired. The change from treated to normal diet was usually made between 8 and 10 A.M. Females were checked once a day (8 A.M.) or sometimes twice a day (8 A.M. and 5 P.M.) for the presence of copulation plugs.

Received January 15, 1960.

[1] The 6α-methyl-17α-hydroxyprogesterone acetate (Provera) was supplied through the courtesy of Dr. H. S. Bryan of the Upjohn Company, Kalamazoo, Michigan.

TABLE 1. NUMBER OF FEMALES, MATED, PREGNANT AND NUMBER OF FETUSES FOLLOWING INGESTION OF PROGESTINS

Dietary progestin	Days from onset of feeding treated diet until:		No. of mice					Mean No. of live fetuses per pregnant female
	Females paired with males	Normal diet resumed	Treated No.	With plugs		Pregnant		
				No.	%	No.	%	
U8839	4	4	15	14	93	11	73	11.6
Progesterone	4	4	13	13	100	12	92	9.8
Progesterone	10	10	14	14	100	11	79	9.8
Progesterone	5	15	30	21	70	20	67	10.9
U8839	5	12	23	18	78	17	74	9.8
Total			95	80	84	71	75	10.4

About 5 days after mating had taken place, the females were removed from the males' cage and again caged in groups. Fifteen days after resuming normal diet, or 11 or 12 days after copulation plugs were observed, all females were autopsied, pregnancy determined, and fetuses and implantation sites counted.

Males were checked for fertility after they had eaten one or the other of the treated diets for up to 25 days by periodically mating them with normal females.

RESULTS AND DISCUSSION

Mating was inhibited from 4 days after starting treatment until about 64 hours after treatment was stopped in all females receiving treated diet. No copulation plugs were found in any of 53 females during 7 or 10 day treatment periods during which they were exposed to males. Time of mating as indicated by the appearance of copulation plugs and checked by correlating fetus size at autopsy, occurred during the third night (64–72 hours) after resumption of normal diet in 71 of 80 females that did mate (Table 1). The other 9 females that mated did so during the fourth night. These 9 were large, older females which had received a treated diet for 12 or 15 days. Larger females may have had larger reserves of the exogenous hormone in the body fat than smaller animals fed for shorter periods of time. The reason for failure of 15 of the 95 treated females to mate is not definitely known. In some cases either the male or the female appeared unhealthy, not necessarily due to the treatment. In some other cases young inexperienced males may have been at fault.

In comparison with mice of similar background in this laboratory, treatment did not appear to adversely affect either the proportion of females becoming pregnant or the number of fetuses present at autopsy. There were no differences between groups of females receiving progesterone or U8839 as indicated by these two measures of fertility (Table 1).

Each mouse ate 3–5 gm. of diet/day and so ingested 18–30 mg. of progesterone or 0.6–1.0 mg. of U8839 per day. The levels of progesterone and U8839 selected for this experiment were chosen on the basis of preliminary testing. Sufficient data are not available to show whether oral effectiveness of the two drugs is necessarily in the ratio fed or that the doses reported are the minimum effective levels.

Fertility and libido of males, which were paired with the females under treatment, did not seem to be affected although some of the males had received one of the treated diets 7 or 10 days before mating. The observation that other males, fed treated diets for 25 days, mated and produced litters of normal size during and after treatment also indicates that the treated diet does not affect males.

On the basis of these experimental findings it appears that *ad libitum* feeding of diets containing either progesterone or U8839 is effective in inhibiting mating in mice and that

synchronization of mating in groups of mice can be effected by simultaneously resuming normal diets.

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THE THYMOLYTIC ACTIVITIES OF 16α, 17α KETALS OF TRIAMCINOLONE[1]

ABSTRACT

The relative thymolytic activities of triamcinolone acetonide and several of its homologous ketonide variants were assessed in a 48–hour bioassay employing intact immature female rats. One of the alkyl substituents of the ketonide moiety was varied in a regular way from methyl through hexyl while the other was held at methyl throughout the homologous series. The methyl, ethyl, propyl, butyl, pentyl, and hexyl analogs were more active than hydrocortisone; however, the latter two were less efficacious than triamcinolone.

The 16α, 17α isopropylidenedioxy derivative of triamcinolone has been reported to possess greater glucocorticoid activity than the parent steroid (1, 2). Moreover, Fried, *et al.* (1) have presented data suggesting a progressive increase in biological activity with decreasing molecular weight of ketal substituents. This communication reports the thymolytic activities of a series of homologous ketals of triamcinolone.

MATERIALS AND METHODS

Intact immature female rats (Sherman strain, 40–60 g.) were given a single subcutaneous injection of steroid suspended in 0.2 ml. of carboxymethylcellulose vehicle (3), with benzyl alcohol excluded. Control animals received vehicle alone. Forty-eight hours after injection the rats were autopsied, at which time thymus and body weights were determined. Results were expressed as thymus weight (mg.)/100 g. final body weight. Statistical computations included analysis of variance (4); determination of relative potencies and 95% confidence limits (5); and calculation of the precision (λ) of a parallel line biological assay (6).

RESULTS

Comparative thymolytic responses to graded concentrations of hydrocortisone, triamcinolone and triamcinolone ketal analogs are presented in Table 1. Though there appears to be a progressive decrease in the relative potencies as the molecular weight (R) increases, no significance can be conferred upon the differences between the methyl (I), ethyl (II) or propyl (III) analogs, as evidenced by the overlap of 95% confidence limits.

A comparison of the relative potencies and 95% confidence limits of a linear 3 carbon structure (III) and a branched 3 carbon structure (IV) indicates that, within this series, configuration does not significantly alter biological activity.

The isobutyl ketal (V) was approximately equivalent to triamcinolone, whereas the addition of another methyl group (VI) resulted in a steroid with an activity less than that

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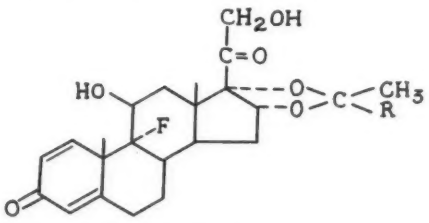
Received February 4, 1960.

[1] This work was conducted in the Departments of Pharmacological Research, Dr. Earl H. Dearborn, Head, and Chemical and Process Development, Dr. G. Sieger, Head.

TABLE 1. THYMUS INVOLUTION DOSE-RESPONSE DATA*



Compound	Total dose (μg./rat)	No. rats	Thymus weight (mg./100 g. final body weight)	Relative potencies and 95% confidence limits	
				(×hydrocortisone)	(×triamcinolone)
Control	—	22	360 ± 13†	—	—
Hydrocortisone	150	31	296 ± 7	1.0	—
	300	32	261 ± 9		
	600	32	199 ± 7		
	900	32	134 ± 7		
	1200	32	111 ± 5		
Triamcinolone	50	20	280 ± 10	4.0 (3.3– 4.8)	1.0
	100	20	234 ± 11		
	200	20	147 ± 8		
	400	20	101 ± 2		
R = CH_3 (I)	20	20	205 ± 9	20.2 (16.6–25.0)	5.1 (4.1–6.3)
	40	20	147 ± 12		
	80	20	107 ± 7		
	160	20	81 ± 4		
R = CH_2CH_3 (II)	25	12	202 ± 14	16.6 (13.2–21.0)	4.2 (3.3–5.4)
	50	12	149 ± 13		
	100	12	98 ± 4		
	200	12	85 ± 5		
R = $CH_2CH_2CH_3$ (III)	25	12	215 ± 12	14.1 (11.3–17.7)	3.6 (2.8–4.5)
	50	12	160 ± 9		
	100	12	110 ± 7		
	200	12	85 ± 6		
R = $CH(CH_3)_2$ (IV)	25	12	221 ± 19	13.0 (10.4–16.2)	3.3 (2.6–4.1)
	50	12	185 ± 19		
	100	12	113 ± 8		
	200	12	88 ± 7		
R = $CH_2CH(CH_3)_2$ (V)	50	12	271 ± 15	4.5 (3.7– 5.6)	1.2 (0.9–1.4)
	100	12	224 ± 15		
	200	12	149 ± 14		
	400	12	93 ± 7		
	800	12	69 ± 4		
R = $CH_2CH_2CH_2CH_2CH_3$ (VI)	100	12	265 ± 14	2.3 (1.8– 2.9)	0.6 (0.5–0.7)
	200	12	203 ± 9		
	400	12	145 ± 13		
	800	12	98 ± 9		
R = $CH_2CH_2CH_2CH_2CH_2CH_3$ (VII)	200	16	190 ± 12	2.7 (2.2– 3.3)	0.7 (0.5–0.9)
	400	16	121 ± 5		
	800	16	86 ± 5		
	1600	16	71 ± 5		

* Pooled Data.
† Mean ± Standard Error.

TABLE 2. ANALYSIS OF VARIANCE OF THYMUS INVOLUTION DOSE-RESPONSE DATA

Line No.	Treatment	Sum of squares	Degrees of freedom	Mean square	Error line	F value	Probability (P)
1	Regression	11.6722	1	11.6722	4	957	<0.01
2	Parallelism	.0528	8	.0066	4	<1	>0.05
3	Non-linearity	.3383	20	.0169	4	1.39	>0.05
4	Within group variance	6.9315	566	.0122			

of triamcinolone. No further depression of thymolytic activity was observed with the hexyl analog.

Thymus weights decreased significantly as the dose of steroid was increased (line 1, $P < 0.01$ Table 2). There was neither a significant departure of dose-response lines from parallelism (line 2, $P > 0.05$), nor a significant deviation of points from the regression lines (line 3, $P > 0.05$). The precision (λ) of the bioassay was 0.23, with a combined slope for compounds of -0.487.

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Acknowledgment

The authors are deeply grateful to Mr. J. Haynes for statistical assistance.

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THE ENDOCRINE SOCIETY

1960 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I. L. Chaikoff in 1958 and Dr. Wilbur W. Swingle in 1959. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator who has not attained his 36th birthday by June 1st of the year the award is presented, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. James A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore

M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, and 1959 Dr. Gordon L. Farrell. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individual possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Scholar of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15, 1959.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the secretary of the Society. They should be submitted by October 15, 1959.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.

THE INTERNATIONAL CONGRESS OF ENDOCRINOLOGY

The First International Congress of Endocrinology will be held in *Copenhagen Denmark, July 18–23, 1960.*

United States members of the International Executive Committee are Dr. Warren O. Nelson and Dr. Leo T. Samuels, Dr. Gregory Pincus is Chairman of the Program Committee. The tentative program consists of ten symposia covering the general field of endocrinology plus round-table discussions and a number of submitted papers. Those desiring to submit papers should immediately contact Dr. Gregory Pincus, Worcester Foundation, Shrewsbury, Massachusetts. There will be a number of interesting scientific and technical exhibits.

In order to facilitate travel arrangements to the Copenhagen Convention, the Council of The Endocrine Society at its San Francisco meeting designated THOMAS COOK & SON as our authorized travel agents. They will in turn offer especially designed All Expense Paid Tours to Europe, including hotel reservations for the convention, as well as offering independent itineraries for any of our members who desire to travel separately by air or by steamship.

Registration is open to anyone interested in endocrinology at an approximate fee of $28 U.S. Members of the families of those attending may register as associates at an approximate fee of $14 U.S., fee to be paid at the time of registration or in advance to the American Express Company, which has been designated by the Executive Committee of the International Congress as official financial and travel agent. There will be a number of social functions and other entertainment provided for all registrants.

As you all know, European travel is unusually heavy during July and it is suggested that those contemplating attending the Congress should start making tentative reservations at the earliest possible moment by filling in and returning the card recently mailed to members, to THOMAS COOK & SON's special representative—Harry E. Kornbaum, Transportation Chairman, Rainbow Travel Service, 2817 Classen Boulevard, Oklahoma City 6, Oklahoma, who will correspond directly with each member about his individual travel arrangements to and from the Convention, at the same time keeping our National Officers fully informed of all travel arrangements of its members. Completion of the card CARRIES NO OBLIGATION OR COMMITMENT ON YOUR PART. Send it in today for the complete travel arrangements for our Society.

The general expenses of a meeting of this magnitude are quite large and

The Endocrine Society has made a substantial contribution from its treasury for organizational purposes. U.S. pharmaceutical firms have also contributed very generously. However, sufficient funds are not as yet available to assure the success of this meeting; therefore, *the committee suggests that each of our members make voluntary contributions to this fund.* Please make your checks payable to: The Endocrine Society—First International Congress of Endocrinology. Mark it *Contribution* and mail it to the office of the Secretary-Treasurer, 1200 North Walker Street, Oklahoma City 3, Oklahoma.

(For program see the December 1959 issue of this Journal.)

THE FOURTH INTERNATIONAL GOITRE CONFERENCE LONDON 1960

Sponsors: The American Goiter Association, The Royal College of Physicians, The Royal College of Surgeons of England, The Royal Society of Medicine, The London Thyroid Club.

Date and Location

The Fourth International Goitre Conference organized jointly by the American Goiter Association and the London Thyroid Club will take place at Church House, Great Smith Street, Westminster, London, S.W.1 (and not at The Royal College of Surgeons as previously announced) on Tuesday, Wednesday, Thursday and Friday, 5th, 6th, 7th and 8th July 1960.

Conference Offices

At 9.30 A.M. on Monday, 4th July, a Reception Office will open at Church House, Westminster, where registration formalities may be completed and where members will receive the various Conference documents, including the final programme. This Office will remain open throughout the Conference. Meantime, all correspondence should be addressed as follows:

The Honorary Secretary
The Fourth International Goitre Conference
The Royal Society of Medicine
Dering Yard
67c New Bond Street, London, W.1

except for registration fees which should be addressed to Dr. F. C. Kelly, 20 Ropemaker Street, London, E.C.2.

JOURNAL OF REPRODUCTION AND FERTILITY

The first number of the new *Journal of Reproduction and Fertility*, official journal of the Society for the Study of Fertility has just recently

become available from the Blackwell Scientific Publications, Oxford, England.

The journal will be published quarterly. Annual subscription post free seventy shillings (U.S.A. and Canada $12.00). The editorial staff is headed by C. R. Austin, Editor and A. S. Parkes, Chairman of the Editorial Board. The journal will publish original papers, abstracts of Proceedings, and occasionally reviews and bibliographies, dealing with the morphology, physiology, biochemistry and pathology of reproduction in man and other animals, and with the biological, medical and veterinary problems of fertility.

Dr. Parkes states in the foreword, "All who work in the field of reproduction and fertility are cordially invited to submit papers, and contributions from abroad will be especially welcome."

FIRST INTERNATIONAL CONGRESS OF HISTOCHEMISTRY AND CYTOCHEMISTRY

PARIS, AUGUST 28–SEPTEMBER 3, 1960

In 1960, from August 28 to September 3, the First International Congress of Histochemistry and Cytochemistry will be held in Paris. It is organized under the auspices of the "Société Française d'Histochimie" in collaboration with the histochemical societies in existence all over the world, especially the "American Histochemical Society," the "Deutsche Arbeitsgemeinschaft für Histochemie," the "Société Belge d'Histochimie," the Italian and Japanese histochemical societies and several non-autonomous sections of histochemistry.

All correspondence on scientific Congress matters should be addressed to:

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The full program will be sent on request.
The registration fee is: $25 for full membership,
$20 for associate members.

THE AMERICAN INSTITUTE OF ORAL BIOLOGY

The 17th Annual Meeting of The American Institute of Oral Biology will be held in Palm Springs, California, October 8–12, 1960, preceding the meeting of the American Dental Association in Los Angeles. The faculty will comprise the following speakers: Benjamin H. Ershoff, John Field, Edgar S. Gordon, Wilton M. Krogman, Balint Orban, C. M. Pomerat, Hamilton B. G. Robinson, and Milton J. Schiffrin.

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ISSUED MONTHLY FOR THE ENDOCRINE SOCIETY
BY CHARLES C THOMAS, PUBLISHER,
SPRINGFIELD, ILLINOIS

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JUN 2 6 1960
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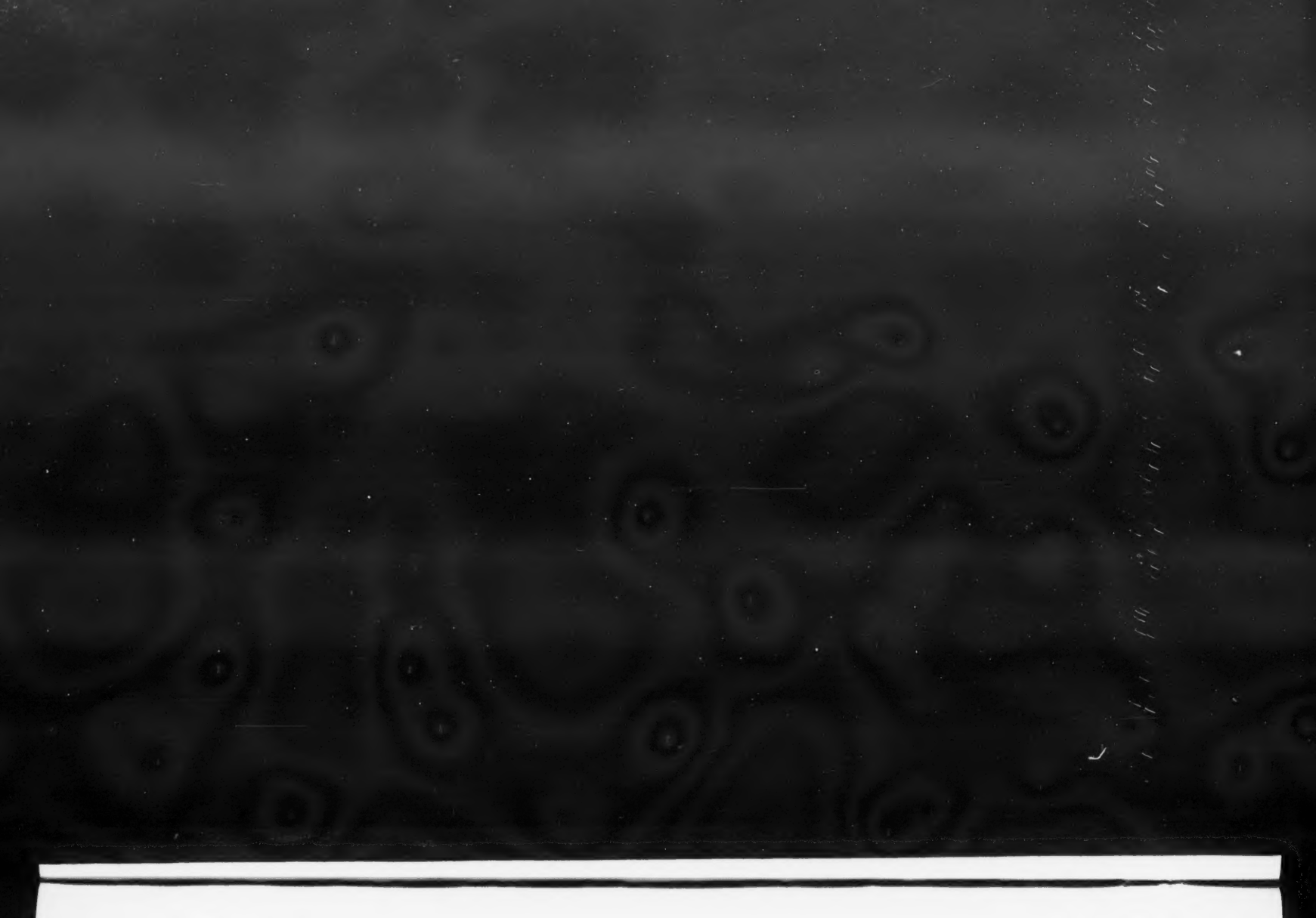
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